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PGE_2 Signaling in Glioblastoma Growth and Invasion

by

HUA TIEN CHEN

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in partial fulfillment of the requirements for the degree of

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in

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Abstract

Glioblastoma multiforme (GBM) is characterized by unrelenting cellular proliferation, exuberant angiogenesis and diffuse tumor cell invasion into the surrounding parenchyma. Recent advances in standard and molecular targeted therapies continue to be a formidable challenge posed by the genetic and cellular heterogeneity within these tumors. This has led to a search for the prevalent targets and novel pathways associated with glioblastoma cell growth, invasion and resistance to current therapies.

The primary objective of this thesis research focuses on the characterization and identification of novel signaling pathways that play critical roles in promoting cell proliferation and invasion in primary glioblastoma cells. Initially we review the recent experimental studies of COX-2/PGE_2 signaling and propose novel therapeutic approaches to glioma treatment. In Chapter 2, utilizing cell-based screening and molecular assays, we present our efforts to validate the biological activity of the dual cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) inhibitors. We show that these compounds induced a G_0/G_1 phase block and apoptosis via a non-COX/LOX mechanism through caspase-3 mediated proteolysis of β-catenin. Our results suggest that compounds having a diarylprop-2-yn-1-one moiety may represent a lead compound for the development of a novel class of chemotherapeutic agents. In Chapter 3, using pharmacological and molecular approaches, we demonstrate that over 50% (7/12) primary cultures of human GBM expressed COX-2 and secreted elevated PGE_2 levels and that COX-

2-derived PGE_2 triggered glioblastoma cell proliferation and invasion mediated through the EP4 via transactivation of the epidermal growth factor receptor and ERK/Akt activation. These results indicate that blocking this pathway may represent a novel therapeutic strategy.

In Chapter 4, protein expression profiling and biochemical analyses show that under hypoxic conditions, PGE_2 modulates the activity of HIF-1α and VEGF induction through the interplay between EP4-mediated HIF-1α, pro-angiogenic cytokines and multiple downstream signaling pathways. Our results support a pivotal role for PGE_2 in initiating the angiogenic switch during cellular transformation and progression, highlighting the complex signaling networks responsible for glioblastoma unrelenting growth and defiant invasion. Furthermore, this thesis work suggests that antagonism of EP4, rather than selective COX-2 inhibitors, may be more efficacious as an alternative approach for the management of these devastating tumors.

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List of Abbreviations

15-PGDH ..... 15-hydroprostaglandin Dehydrogenase

5-HETE ..... 5-Hydroperoxyeicosatetraenoic Acid

5-LOX ..... 5- Lipoxygenase

AA ..... Arachidonic Acid

APC ..... Adenomatous Polyposis Coli

BBB ..... Blood Brain Barrier

BCA ..... Bicinchoninic Acid

bFGF ..... Basic Fibroblast Growth Factor

cAMP ..... Cyclic Adenosine Monophosphate

CDDP ..... Cisplatin

CdKs ..... Cyclin-dependent kinases

CHX ..... Cycloheximide

CNS ..... Central Nervous System

COX-1 ..... Cyclooxygenase-1

COX-2 ..... Cyclooxygenase-2

CXB ..... Celecoxib (Celebrex)

CXCR-4 ..... Chemokine (C-X-C Motif) Receptor 4

CysLTs ..... Cysteinyl Leukotrienes

DMC ..... Dimethyl Celecoxib

DMEM ..... Dulbecco's Modified Eagle's Media

DMSO ..... Dimethyl Sulfoxide

ECM ..... Extracellular Matrix

EGF ..... Epidermal Growth Factor

EGFR ..... Epidermal Growth Factor Receptor

EGR-1 ..... Early Growth Response -1

ELISA ..... Enzyme Linked Immunosorbent Assay

EP1 ..... E-prostanoid-1 receptor

EP2 ..... E-prostanoid-2 receptor

EP3 ..... E-prostanoid-3 receptor

EP4 ..... E-prostanoid-4 receptor

EPC ..... Endothelial Progenitor Cell

ERK1/2 ..... Extracellular Signal-Regulated Kinase 1 and 2

FBS ..... Fetal Bovine Serum

FDA ..... US Food and Drug Administration

GBM ..... Glioblastoma Multiforme

GPCRs ..... G-Protein-Coupled Receptors

GSK-3β ..... Glycogen Synthase Kinase-3β

HB-EGF ..... Heparin Binding Epidermal Growth Factor

HIF-1 ..... Hypoxia-Inducible Factor-1

HIF-1α ..... Hypoxia-Inducible Factor-1 Alpha

HRE ..... Hypoxia Response Element

IC_{50} ..... Inhibition Concentration at 50%

IDH ..... Isocitrate Dehydrogenase

IGF ..... Insulin-like Growth Factor

IGFBP-3 ..... Insulin Growth Factor Binding Protein -3

IL-6 ..... Interleukin-6

IL-8 ..... Interleukin-8

JNK ..... c-Jun NH_2-Terminal kinase

LOH ..... Loss of Heterozygosity of Chromosome

LTs ..... Leukotrienes

MAPK ..... Mitogen-Activated Protein Kinase

MB ..... Medulloblastoma

MCP ..... Monocyte Chemotactic Protein-1

MGMT ..... O_6-Methylguanine-DNA Methyltransferase

MMP-9 ..... Matrix Metalloproteinase Protein-9

mPGES-1 ..... Microsomal Prostaglandin E Synthase-1

mTOR ..... Mammamlian Target of Rapamycin

NDGA ..... Nordihydroguaiaretic Acid

NF-κB ..... Nuclear Factor-kappa B

NHA ..... Normal Human Astrocyte

NSAIDs ..... Nonsteroidal Anti-Inflammatory Drugs

PBS ..... Phosphate Buffer Saline

PDGF ..... Platelet-Derived Growth Factor

PDK-1 ..... 3-Phosphoinositide-dependent Kinase-1

PGE_2 ..... Prostaglandin E_2

PGG_2 ..... Prostaglandin G_2

PGH_2 ..... Prostaglandin H_2

PGs ..... Prostaglandins

PI3K ..... Phosphatidylinositol 3-Kinase

PPARδ ..... Peroxisome Proliferation Activated Receptor-δ

PTEN..... Phosphatase and Tensin homolog on Chromosome 10

RT-PCR ..... Reverse Transcriptase Polymerase Chain Reaction

SAR ..... Structure Activity Relationship

siRNA ..... Silence Interference Ribonucleic Acid

TCF-4 ..... T Cell Factor-4

TGF-β ..... Transforming Growth Factor-β

TIMPs ..... Tissue Inhibitor of Metalloproteinases

TMZ ..... Temozolomide

TNF-α ..... Tumor Necrosis Factor-α

TTBS ..... Tris-Tween Buffered Solution

TXB_4 ..... Thromboxane B_4

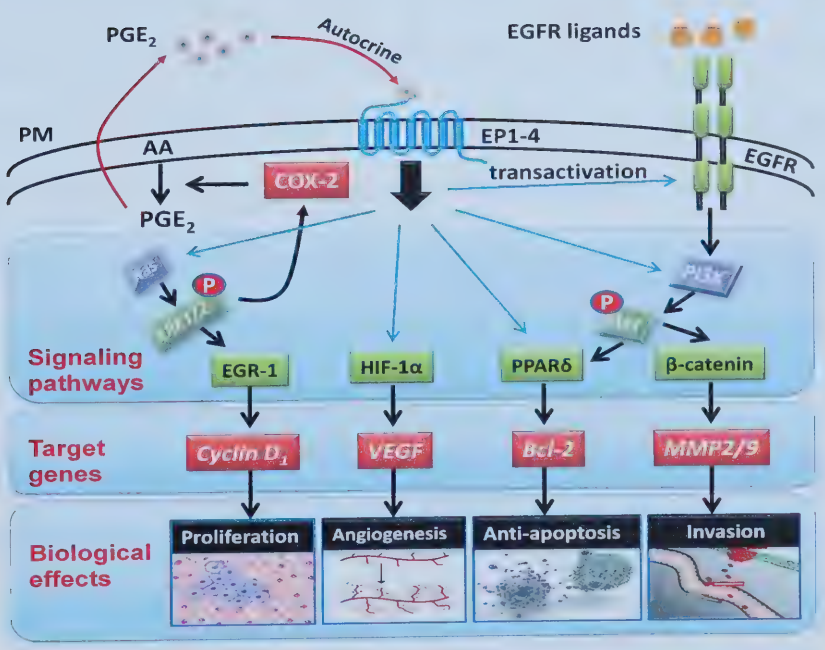
TXBs ..... Thromboxanes

VEGF .....Vascular Endothelial Growth Factor

VP16 ..... Ectoposide

Chapter 1

Arachidonic Acid Metabolism and the Influence of Prostaglandin E_2 on Glioma Biology and Treatment



1.1. Introduction

Gliomas are primary tumors of the central nervous system consisting of cells displaying morphological features toward glial differentiation. The World Health Organization classifies these tumors morphologically which display features of astrocytic, oligodendroglial, or ependymal cells (1) and grades these tumors histologically on a scale of I to III/IV with increasing malignancy and poor clinical outcome (2). Grade I (pilocytic astrocytoma) and grade II (diffuse astrocytoma) tumors are low-grade malignancies with a slow growing phenotype and better clinical outcome; grade III (anaplastic astrocytoma) and grade IV (glioblastoma) are highly malignant with fast growing invasive phenotypes and poor clinical outcomes. Unlike other solid tumors, gliomas rarely metastasize outside the CNS but have a remarkable tendency to infiltrative surrounding normal brain tissue, complicating surgical resection (3). The annual incidence of malignant gliomas is less than 5 cases per 100,000 people in which glioblastoma multiforme tumors (GBMs) constitute 60-70% of malignant gliomas (4). Primary malignant gliomas are 1.5 times more common in men than women, whereas the reverse is the case for secondary GBM (5). There is some evidence that Caucasians are more frequently affected than their African or Asian counterparts (6). The prognosis for patients with newly diagnosed malignant glioma remains dismal. Despite aggressive treatment with multiple therapeutic modalities including surgery, radiation therapy and various chemotherapies, the median survival is 12-15 months for grade IV astrocytoma (GBM) patients and 2-5 years

for grade III anaplastic astrocytoma patients from diagnosis (7) -- a statistical fact that has changed little over the past four decades. Tumor recurrence is invariably universal even after multimodality therapy. Consequently, novel treatment strategies such as molecularly targeted therapies directed against tumor proliferation and invasion are urgently needed.

Although the etiology and predisposing factors of GBM are poorly understood (8, 9), exposure to ionizing radiation has been proven to be a strong risk factor for gliomas, as seen in children previously irradiated for leukemia (10). Interestingly, higher socioeconomic status tends to increase the risk for gliomas (11). Nevertheless, GBMs presumably originate from astrocytic lineages and consist mainly of poorly differentiated transformed astrocytes. Diagnosis of GBM is based primarily on two main histopathological features of microvascular proliferation and pseudopalisading necrosis along with nuclear atypia and high cellular and mitotic activity (4). Based on biological and genetic differences, GBMs have been further subdivided into two main subtypes (**Figure 1-1**) (5, 12). Primary GBMs, also defined as "*de novo*", tend to occur in patients over 50 years of age (13) and are characterized with amplification and constitutive activation of the epidermal growth factor receptor (*EGFR*), deletion of the phosphatase and tensin homologue on chromosome 10 (*PTEN*), *TP53* mutation and loss of heterozygosity (LOH) of chromosome 10q (5). Secondary GBMs are typically observed in younger patients who initially present as low-grade or anaplastic astrocytomas and progress into GBM over a period of 5-10 years (13); these tumors, occurring much less than 10%, are characterized by *TP53* mutation, LOH

10q (5, 12, 14), *IDH1* mutation (15-17) and overexpression of the platelet-derived growth factor (PDGF) or its receptors (18). Both types of GBMs are morphologically and clinically indistinguishable (19) and often respond similarly and poorly to aggressive treatment with multiple modalities but may respond differently to molecularly targeted therapies that exploit the tumors genetic and molecular signatures.

Recently, there has been an exponential explosion in knowledge of the biology and genetics of malignant gliomas, which may provide a gateway for the development of novel therapeutic strategies. A plethora of profiling and genomic data indicates that gliomas comprise a complex of multistage genetic alternations and chromosomal abnormalities such as deletions, amplifications, loss of heterozygosity, and epigenetic modulations such as DNA methylation or histone acetylation (7, 20, 21). The most common defects in gene alteration and epigenetic change involve *EGFRvIII*, a constitutively autophosphorylated genomic deletion variant of *EGFR* (22) and O_6-methylguanine-DNA methyltransferase (MGMT), an enzyme that repairs mismatch DNA damage (23). Recent studies have found that coexpression of *EGFRvIII* gene deletion and *PTEN* wild type gene provides good responsiveness to EGFR kinase inhibitors in GBM cells, whereas epigenetic silencing of the *MGMT* gene by promoter methylation enhances temozolomide (TMZ) chemotherapy in GBM patients. Furthermore, molecular genetic analysis identified allelic LOH 1p and 19q co-deletion in patients with anaplastic oligodendrogliomas as predictor of chemotherapeutic response (24). Although the molecular genetics approaches may

aid therapeutic decisions and help predict clinical outcome in GBM patients, the overall survival of responsive patients has increased marginally in the last 20 years (10). GBMs recur after standard (i.e. radiation, concomitant and adjuvant TMZ) treatment due to an unrelenting ability to infiltrate diffusely into surrounding normal brain parenchyma. Thus, development of effective anti-invasion therapy remains a high priority for future therapies.

In addition to the molecular genetic abnormalities and epigenetic changes that occur during cellular transformation in gliomas, another important factor which allows these tumors to grow and invade into the surrounding normal brain tissue is the establishment of a new tumor vasculature through the process of angiogenesis (25). In 1971, Judah Folkman initially proposed the concept of tumor-associated angiogenesis, and he suggested that the inhibition of angiogenesis, and thus denying the cell of oxygen and nutrients, would be an effective way to inhibit tumor cell growth and proliferation (26). After 33 years, his theory of anti-angiogenic therapy was validated in 2004 when the first drug bevacizumab, an anti-VEGF humanized monoclonal antibody was developed as inhibitor of angiogenesis for the treatment of metastatic colorectal cancer. Bevacizumab approved by the US Food and Drug Administration (FDA) in 2004 (27), was recently approved for second-line treatment of GBM based on questionable survival benefit and less toxicity in a Phase II trial of grade III and IV astrocytomas (28-31). Contrary to initial expectations, the survival benefits observed in patients with recurrent malignant gliomas when used in combination with conventional chemotherapy remain questionable at present. Inevitably GBMs

recur after anti-VEGF targeted therapy. Preclinical evidence suggests that anti-VEGF therapy may promote glioma cells to co-opt host blood vessels (without angiogenesis) and allow the cells to grow as a more invasive phenotype (32-36). Thus, it is imperative to develop more efficacious novel therapeutic strategies that target key molecular pathways responsible for glioma cell growth and invasiveness.

1.2. Overview of Arachidonic Acid Metabolism

Genetic, pharmacological, and epidemiological studies have corroborated the pivotal role of cyclooxygenase-2 (COX-2) in tumor angiogenesis and tumorigenesis (37, 38). In transgenic mouse models, overexpressed *COX-2* in mammary gland or basal keratinocytes developed focal mammary gland or epidermal hyperplasia and dysplasia, respectively (39, 40). Targeting COX-2 with selective COX-2 inhibitors was found to prevent colorectal neoplasia (41), reduce tumor growth and metastases in various types of cancers (42). These studies suggest that COX-2 may be a novel molecular target for the prevention and treatment of cancers including gliomas.

The remainder of this chapter will address and review arachidonic acid metabolism pathways, discuss the recent advances in understanding the mechanisms for COX-2 and COX-2-derived prostaglandin E_2 (PGE_2) in tumorigenesis, and will focus on the molecular mechanisms which operate

between PGE_2 and other key survival signaling pathways that modulate GBM cell growth and invasion. A more comprehensive understanding of these pathways may lead to better treatment strategies against glioblastomas.

Arachidonic acid (AA) is an essential component of cell membrane phospholipids that is esterified via the action of membrane-bound phospholipase A_2. The released AA can be metabolized by three enzymatic pathways (**Figure 1-2**): the two well characterized cyclooxygenase (COX) and lipoxygenase (LOX) pathways and the less recognized cytochrome p450 pathway. In the COX pathway, the rate-limiting enzymes COXs oxidize the free AA to form an unstable intermediate prostaglandin G_2 (PGG_2), which is then rapidly converted to prostaglandin H_2 (PGH_2) by the hydroperoxidase activity of COXs. PGH_2 is thereafter isomerized by various tissue specific prostaglandin synthases into a series of biologically active metabolites of prostaglandins (PGs) such as PGE_2 and thromboxane B_2 (TXB_2) (43). Three isoforms of COX have been identified for their distinct expression patterns and biological activities. *COX-1* (chromosome 9) is constitutively expressed in nearly all normal tissues and is responsible for regulation of cellular homeostasis (44). *COX-2* (chromosome 1) can also be constitutively expressed in discrete populations of neurons and astrocytes (45), kidney (46) and encodes an inducible COX-2 enzyme which is markedly upregulated by a variety of inflammatory and mitogenic stimuli, including interleukin 1α/β (IL-1α/β), tumor necrosis factor α (TNF-α) (47), epidermal growth factor (EGF) (48, 49), chemotherapeutic drugs such as taxane and paclitaxel (50) and cellular stresses such as hypoxia and radiation (51, 52). *COX-3*

(chromosome 9), a splice variant of *COX-1*, is expressed in high levels within the human cerebral cortex and is potently sensitive to nonsteroidal anti-inflammatory drugs (NSAIDs) (53, 54). However, COX-3 is not induced by acute inflammatory stimuli (55) and the biological function of COX-3 remains largely unknown.

In the LOX pathway, the free AA is also metabolized by three major types of lipoxygenases—5-LOX, 12-LOX, and 15-LOXs, to form diverse leukotrienes (LTs). 5-LOX, especially 5-hydroperoxyeicosatetraenoic acid (5-HETE) is further metabolized to leukotriene B4 (LTB4), cysteinyl leukotrienes LTC4, LTD4 and LTE4 (collectively called CysLTs) (56). Unlike COXs, LOXs are expressed in a cell type-specific fashion. For example, 5-LOX is constitutively expressed in cells of myeloid lineage and pulmonary artery endothelial cells (57). Similarly, 12-LOX, commonly found in the brain (58), is also expressed predominantly in platelets (59) whereas 15-LOX is found in haemoglobin and in reticulocytes during anemia (60, 61). 5-LOX, one of the most important members of the LOX family, can be induced by transforming growth factor β and 1,25-dihydroxyvitamine D_3 (62) and has been found to be overexpressed in a variety of cancer including brain tumors (63, 64). LTs have been shown to be important lipid mediators linked to cerebral ischemia, brain edema, and increased permeability of the blood-brain barrier in brain tumors (65). Although very limited data are available on the 5-LOX metabolic pathway, the efficacy of 5-LOX inhibitors has been shown in preventing lung carcinogenesis by reducing tumor multiplicity and tumor incidence in mice by 75% and 20%, respectively (66).

Several studies have reported that COX-2 and 5-LOX are frequently coexpressed and upregulated in several types of cancer cell lines and tumor specimens (67, 68) and have been implicated in promoting tumor cell growth and angiogenesis via the induction of VEGF expression (69-71). Because both COX-2 and 5-LOX share similarities in biological function and convergent targets, it is likely that inhibition of either COX-2 or 5-LOX pathways alone may activate the other. In fact, one study showed that treatment with a combined COX-2/5-LOX inhibitor could prevent either COX-2 or 5-LOX "compensatory" pathways and had an additive effect in inhibiting cell proliferation (68). Therefore, dual COX-2 and 5-LOX inhibitors may represent promising therapeutic approaches in cancers which are dependent on COX-2/5-LOX metabolism.

In addition to COX and LOX pathways, the cytochrome P450 monooxygenase pathway (not shown) is involved in the conversion of AA into biologically active metabolites by an NADPH-dependent oxidative reaction (72). Various isoforms of cytochrome P450 have been shown to be present in discrete regions of the CNS in the mouse (73). However, little is known about the role of cytochrome P450 enzyme and its metabolites in cancer progression.

1.2. The Role of COX-2 in Cancer

1.2.1. Overexpression of COX-2 in Cancer

Epidemiological studies demonstrate that COX-2 is overexpressed in many premalignant and malignant human tissues (**Table 1**) including low grade astrocytomas (30%) and GBM (71-74%) (74) and that long-term use of classical NSAIDs over a 10-15 year period such as aspirin, a well known target of COX enzymes, can reduce the risk of cancer development by 40-50% (75), indicating that inhibition of COXs has a chemopreventive effect. A genetic study using a gene-knockout mouse model for human Familial Adenousdous Polyposis (FAP) has shown that COX-2 induction plays a critical role in intestinal tumorigenesis (76). Disruption of *COX-2* gene in $Apc^{\Delta 716}$ mice markedly suppressed intestinal polyp formation (77). Moreover, a direct role for COX-2 in tumorigenesis was studied in *COX-2* transfected multiparous female transgenic mice which demonstrated that enhanced COX-2 expression was sufficient to induce mammary gland hyperplasia, dysplasia and transformation into mammary tumors (39).

In addition to the epidemiological and genetic studies noted, recent pharmacological investigation has suggested that COX-2 inhibition may be a potential therapeutic target. The selective COX-2 inhibitor celecoxib (Celebrex; 1,5-diaryl pyrazole-based compound) (78) inhibited tumor cell growth *in vitro* and reduced formation of colorectal, gastric, oesophageal, hepatocellular, pancreatic, lung, breast, skin, prostate, and bladder tumors in xenograft rodent models (79), supporting the hypothesis that celecoxib may have clinical application in cancer prevention and treatment. Further, pharmacological data are required to support the assumption that COX-2 represents a potential therapeutic target for GBM chemoprevention and treatment. Although *in vitro* or *in vivo* studies in mice

showed great promise for celecoxib as a GBM radiosensitizer and inducer of prolonged survival in GBM xenograft mice models (80), clinical studies in GBM patients have demonstrated that celecoxib combined with radiation (81), or chemotherapeutic agent TMZ and angiogenic agent thalidomide (82) did not appear to show any overall survival benefit in patients with newly diagnosed GBM.

1.2.2. COX-2-derived PGE_2 Involves Tumor Progression

The molecular basis for the anti-inflammatory and anti-tumor effects of celecoxib is in part related to its inhibition of COX-2 enzyme activity, and the subsequent decrease in production of PGE_2, a major metabolite of the COX-2 pathway. Several studies support the notion that the chemopreventive actions of NSAIDs and celecoxib are dependent on the attenuation of PGE_2 levels. In one study (APC^{min} mouse model-which carries an inactivating mutation adenomatous polyposis coli (*APC*) gene), exogenously added PGE_2 protected small intestinal adenomas from NSAID-induced regression (83) whereas in a second study treatment of APC^{min} mice with PGE_2 accelerated the growth of intestinal adenomas (84). Furthermore, a recent study in medulloblastoma (MB), a common malignant brain tumor of childhood, showed that treatment of medulloblastoma xenografts with selective COX-2 inhibitor celecoxib significantly inhibited MB cell growth, increased apoptosis and suppressed angiogenesis (85), highlighting

the important role of PGE_2 for MB growth. Interestingly, overexpression of COX-2 and increased production of PGE_2 are frequently observed in human brain tumor tissues and as well as numerous types of tumors (74, 86-88). In addition, overexpression of COX-2 in GBM correlates well with tumor grade and poor prognosis (86, 89, 90). Another study showed that elevated levels of PGE_2 was risk factor for brain tumor growth and enhanced invasive potential (91). These studies provide evidence that PGE_2 plays a critical role in tumor development and progression in gliomas and that the lipid-signaling molecule PGE_2 may serve as a novel target for chemopreventive and therapeutic intervention by NSAIDs or related compounds.

1.3. Inflammation in the Brain

For many years, the brain was considered as an "immune privileged "organ, which possessed a decreased susceptibility to inflammation and was unaffected by immune responses. However, this dogma has been revised significantly as many reputable studies have demonstrated that the brain exhibits local inflammation in response to viral and bacterial infections, ischemic injury or neurodegenerative disease (92, 93). Such inflammatory processes generate local inflammatory mediators released from the injured tissue, activated microglia or infiltrating inflammatory polymorphonuclear leukocytes and lymphocytes derived from the blood stream. Normally, neuroinflammation serves as neuroprotective mechanism

associated with repair and tissue remodelling (94) but sustained or excess neuroinflammation can become detrimental to healthy brain tissue (95), causing numerous neurological diseases such as multiple sclerosis, Alzheimer's disease, Parkinson's disease and ultimately neoplastic development.

The link between inflammation and cancer is not new, as first proposed more than a century ago by Rudolf Virchow. He hypothesized that the chronic inflammation site was a cofactor for origin of cancer development. To date, chronic inflammation is well recognized as a risk factor for neoplastic development and accounts for ~15% of malignancies worldwide (96). Chronic inflammation has been postulated to promote tumor growth which is mediated in part through angiogenesis by up-regulation of an array of various proinflammatory cytokines, including TNF and members of its superfamily, IL-1α/β, IL-6, IL-8, matrix metalloproteinase protein-9 (MMP-9) and VEGF. These induced factors influence the expression of proinflammatory mediators such as COX-2-derived PGE_2, and other proinflammatory cytokines and chemokines. It is the profile of cytokine and chemokine balance in the tumor microenvironment that plays a critical role for the development of chronic disease or cancer progression (97, 98). For example, an abundance of the proinflammatory cytokine IL-17 produced by a T-cell subset (Th 17) is mediated by the action of PGE_2 which tilts the cytokine/chemokine balance towards IL-12/IL-23 in the local inflammatory milieu to favour Th17 cell development and IL-17 production (99-101).

1.4. The Role of PGE_2 Signaling in Cancer

1.5.1. PGE_2 and Prostaglandin E Receptors Signalings

PGs are short-lived small lipid autocoids involved in diverse host physiological and pathological processes such as kidney function, inflammation, immunity, neurotransmitter release and carcinogenesis (102-105). Once synthesized, PGs are quickly released from cells and act as autocrine or paracrine signaling mediators by binding to specific, high-affinity cell surface receptors (106). One of the most studied PGs is PGE_2. PGE_2 is produced by many cells types in the body such as macrophages, fibroblasts and some types of malignant cells including gliomas. Among the COX metabolites, basal brain PGE_2 is the most abundant eicosanoid produced by nearly all cells in the CNS and is regulated by COX-2 via NF-κB pathway (107). PGE_2 is also involved in numerous physiological processes in the brain including synaptic plasticity, long-term potentiation, neurogenesis, mediators of pyrogenesis (108-111), pain, fever and immune responses (112, 113). PGE_2 causes increased vasopermeability and vasodilation and is considered to be a mediator of peritumor brain edema in GBM's (114). In the steady state, PGE_2 levels are maintained by the actions of membrane-bound prostaglandin E synthases (mPGESs) to produce active PGE_2 and 15-hydroprostaglandin dehydrogenase (15-PGDH), a degrading enzyme which breaks down PGE_2 into the inactive 15-keto PGE_2 metabolite (115, 116).

Notably, overexpression of mPGES and loss of 15-PGDH were found in colorectal adenoma and colorectal cancers (117, 118), lung cancers (119, 120) and breast cancers (121, 122). Recently, overexpression of prostaglandin synthases including mPGES-1, PGES-2 and cPGES were also found in human gliomas (123) and the expression of 15-PGDH was observed to have tumor-suppressor properties in glioma cell lines (124). These findings underscore the oncogenic potential of the PGE_2 signaling pathway.

PGE_2 exerts its diverse biological activities by binding to a subfamily of unique cell surface G-protein-coupled receptors (GPCRs) namely EP1, EP2, EP3 and EP4 (125, 126) which are differentially expressed on neuronal and glial cells (microglia and astrocytes) (127). For example, EP3 receptors are constitutively expressed in neurons throughout the brain whereas EP1, EP2 and EP4 receptors have been found only in glial cell groups (128, 129). EP1 receptor appears to be the least expressed amid the receptor subtypes (130). These multiple receptor subtypes are distinguished from one another by their differential ligand affinity to PGE_2 and by their unique coupling to opposing second messengers systems through guanine nucleotide-binding protein (G proteins), thereby allowing for potential synergism or antagonism actions in tissues and cells (131, 132). Activation of the EP1 receptor results in an increase of intracellular calcium levels through a Gq/p protein signal mediated by phospholipase C (133) while stimulation of the EP2 receptor promotes the production of intracellular cyclic AMP through Gs proteins (134, 135). Activation of the EP3 receptor, which is expressed as three different isoforms (EP3α, β and γ), inhibits cAMP signaling

primarily through Gi proteins (132, 136) whereas stimulation of the EP4 receptor triggers both the activities of cAMP and phosphatidylinositol 3-kinase (PI3K) signaling pathway via Gi proteins (137). In humans, all of the four EP receptor subtypes respond to PGE_2 with binding affinities in the following sequence of order : EP3 (k_d=0.3nM) > EP4 (k_d=0.8nM) > EP2 (k_d=4.9nM) > EP1 (k_d=9.1nM) (138); each with its own cell-specific difference in expression and signaling activity. Thus, it is not surprising that PGE_2 may activate more than one subtype of EP receptors.

1.5.2. PGE_2 and Prostaglandin Receptors Promote Tumorigenesis

Although the precise role of each individual receptor subtype in tumor development has not been fully characterized, recent results from specific EP receptor knockout mice studies indicate that all four prostanoid receptors are associated with tumor growth, migration and invasion, anti-apoptosis and angiogenesis in colon cancer and other neoplasms (**Figure 1-3**) (139-141). For example, the *Apc* gene mutated mice (Apc^{min} mice) with knockdown of the EP2 receptor exhibited a reduction in angiogenesis by 30% and a decrease in the growth and number of polyps (142, 143). Similarly, COX-2-derived PGE_2 increased VEGF expression via the EP2 receptor in human pancreatic cells (144). Growth of xenografts derived sarcoma 180 and Lewis lung carcinoma in EP3 null mice was markedly hampered (145). Furthermore, Apc^{min} mice treated with a

colon carcinogen, azoxymethane, aberrant crypt foci formation was suppressed by 60% in both the EP1 receptor and EP4 receptor null mice (146, 147).

Other studies have reported that PGE_2 promotes tumor cell growth through activation of EP receptors via multiple downstream targets and signaling pathways. For instance, the tumor growth-promoting effect of PGE_2 was shown to be associated with EP2/EP4 receptors via the activation of PI3K and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathways as well as the β-catenin/T-cell factor transcriptional activity in colon adenocarcinoma (137, 148, 149). Similarly, PGE_2 stimulation in colorectal cancer cells caused translocation of β-catenin to the nucleus and activated transcriptional Wnt target genes such as *c-myc, cyclin D1* and *VEGF* (150, 151). In one study, it was demonstrated that PGE_2 treatment via EP2 receptor led to a direct association of the Gα subunit with the regulator of G protein signaling axis. This binding caused inactivation of glycogen synthase kinase 3β (GSK-3β), leading to accumulation of β-catenin in the nucleus (150). Another study reported that PGE_2 increased GSK-3β phosphorylation, thereby preventing β-catenin degradation and leading to increased β-catenin /T cell factor (TCF) transcriptional complex in the nucleus (151). In addition to these pathways, PGE_2 was hypothesized to indirectly activate peroxisome proliferation-activated receptor δ (PPARδ) in a PI3K/Akt growth-dependent fashion in APC^{min} mice. The effect of PGE_2 was abrogated in the mice that lacked the PPARδ receptor ($PPAR\delta^{-/-}$) (84). Furthermore, PGE_2 stimulation of the EP4 receptor has also been shown to stimulate rapid transactivation and phosphorylation of EGFR via an

intracellular mechanism mediated through the β-arrestin 1/c-Src complex (152). This pathway was shown to play a pivotal role in colorectal cancer cell migration and metastatic spread to the liver. Taken together, these studies provide evidence that PGE_2 signaling promotes tumor growth and angiogenesis, depending on the differential expression of individual receptor subtypes in different tissues and cells. Subsequent elucidation of the biochemical properties and expression patterns of the various subtypes of EP receptors on cells will contribute to the development of potential anti-glioma therapeutic compounds.

Considerable evidence supports the premise that the EP4 receptor is a prominent signaling node which mediates the function of PGE_2 in tumor cell growth, migration, invasion and metastasis in breast and colon cancers (152, 153). Studies have shown that blockade of EP4 signaling with small-molecule EP4 antagonists attenuated cell proliferation as well as breast cancer cell metastasis *in vitro* (154, 155). Forced expression of EP4 receptor also promoted cell growth and anchorage-independent growth (156). However, very limited information is available regarding the functional role of EP receptors in brain cancers. Recent studies have reported that the expression of EP4 is correlated with tumor cell growth in GBM via Sp-1 transcription factor or by PGE_2-dependent activation of type II protein kinase A, (157, 158). In addition, EP4 receptor upregulation was mediated by PGE_2-induced IL-6 release via p38 MAPK pathway in rat astrocytes and human astrocytoma cell lines (159). Statistical analysis based on microarray and immunohistochemical studies revealed that EP4 receptor was overexpressed in GBM but not in anaplastic astrocytoma and that EP4 receptor expression

significantly correlated with the degree of intratumor necrosis and patient survival in GBM (160), demonstrating that the EP4 receptor may be a critical regulator of GBM tumorigenesis and progression.

1.5.3. PGE_2-mediated Signaling Pathways in Angiogenesis and Invasion

PGE_2 has been found to be a potent inducer of the angiogenic switch (161) by tilting the balance toward upregulated expression of its downstream proangiogenic factors targets such as VEGF and basic fibroblast growth factor (bFGF). Interestingly, VEGF and bFGF induce COX-2 expression in endothelial cells and fibroblasts (162, 163), thereby elevating PGE_2 production which upregulates COX-2 expression in a positive autocrine loop via EP receptors through the ERK/MAPK pathway in intestinal adenomas (88). Moreover, PGE_2 promotes angiogenesis via upregulation of chemokine (CXC motif) receptor 4 (CXCR-4) mediated by the induced activity of VEGF and bFGF (164). CXCR-4 has been shown to be highly expressed and involved in cell migration and invasion in GBM (165-167), breast cancer (168), prostate cancer (169) and pancreatic cancer (170). Likewise, elevated levels of COX-2-derived PGE_2 has been found to promote tumor cell growth by upregulated COX-2 expression in a positive autocrine loop via EP receptors through the ERK/MAPK pathway (88) or the hypoxia-inducible factor-1α (HIF-1α) mediated pathway (171) in colorectal tumor cells. In addition, forced expression of COX-2 in colon cancer cells

triggered cell growth via the induction of EGFR (172) and promoted angiogenesis via the increased levels of VEGF (173), implicating a cross-talk mechanism which exists between these two pathways. Indeed, ample evidence indicates that PGE_2 not only binds to its cognate receptors but also transactivates (cross-talk) EGFR (174, 175), PPARδ (84, 176) and Wnt/β-catenin (150, 151), IL-6/STAT3 (177, 178) signaling pathways to stimulate cell growth, induce angiogenesis, migration and invasion. In summary, these studies suggest that blockade of PGE_2 signaling or PGE_2-mediated signaling pathways may provide an alternative therapeutic strategy directed against several types of cancers including gliomas.

In addition to COX-2/PGE_2 modulation of autocrine and paracrine loop signaling pathways, it was shown that forced expression of COX-2 alone led to increased invasiveness in colon cancer and increased metastatic potential of breast cancer to lung (179, 180). Although the mechanism(s) by which COX-2 promotes tumor cell invasion has not been clearly elucidated, the levels of COX-2 expression has been found to significantly correlate with the degree of biological aggressiveness, i.e. tumor grade, proliferative potential and the expression of VEGF, in patients with GBM when compared patients with low grade glioma or with non-tumor patients (89, 91, 181, 182). In addition, high expression of COX-2 and high concentrations of PGE_2 has been seen in other tumors of CNS origin including ependymoma and meningioma (86, 89, 91, 181, 183, 184). Altogether, these studies reinforce the view that COX-2 or the COX-2-derived PGE_2 plays a critical role in influencing the development of an invasive phenotype in CNS

tumors which warrants further investigation of PGE_2 targeting with antagonists of EP receptors as a novel therapeutic approach against GBM's.

Angiogenesis is a prerequisite for solid tumor outgrowth beyond 1 to 2 mm (185), invasion and metastasis (186, 187). This process is mainly driven by the VEGF signaling, which stimulates endothelial cell proliferation (188). However, infiltrative glioma cell growth may initially occur by an angiogenesis-independent mechanism via vessel co-option (189, 190) or through a stem-cell like phenotype (191). In the process of co-option of host vessels, glioma cells grow initially along pre-existing blood vessels to infiltrate white matter tracts and cortical areas (190) and thereafter inducing angiogenesis sprouting from vessels at the tumor periphery (189). In the stem-cell like model, undifferentiated stem cells adapt the growth characteristics of angiogenic cells that progress to become vascular endothelial cells capable of inducing angiogenesis (191).

GBM tends to recur at high frequency (90%) within 2 cm of the primary site after standard care of treatments (192). Although extremely invasive and highly vascularised, GBM rarely display distant metastases. Glioma invasion is a complex process which in part involves the degradation of extracellular matrix (ECM) components (laminin, fibronectin, and type IV collagen) by numerous matrix metalloproteinase's proteins (MMPs), notably MMP-2 and MMP-9 (193, 194). Glioblastoma cells secret high levels of MMP-2 and MMP-9 as pro-enzymes, which require further proteolytic cleavage for activation by ADAMs (a disintegrin and metalloproteinase). High levels of MMPs correlate well with tumor grade (194, 195). Epidermal growth factor (EGF) is the best inducer of

GBM invasion. Upon binding to EGFR, EGF upregulates MMP-9 expression via the activation of ERK and PI3K signaling pathways (193). Current strategies to inhibit MMP activity have focussed on the endogenous inhibition of tissue inhibitor of metalloproteinases (TIMPs). Although TIMP-2 has a profound effect on glioma invasion, it also paradoxically promotes invasion behaviour by upregulating MMP-2 (196). Several excellent recent reviews on glioma cell invasion have been published (3, 193, 197, 198) and will not be reviewed in this chapter. Nevertheless, GBM recurrence risk after radio-and chemo-therapies is largely due to the continued growth of highly invasive resistant cells or glioma stem cells that have already invaded into surrounding brain parenchyma (199). This type of invading glioblastoma cells is likely triggered by autocrine loop signals transmitted through secreted mitogenic factors via receptors on the tumor cells (200). For example, the mutant *EGFRvIII* expressing GBM cells induce upregulation of a number of cytokines and growth factors such as transforming growth factor-α (TGF-α), heparin-binding EGF (HB-EGF) and IL-8, all of which are linked to some aspect of cell proliferation, angiogenesis and invasion via an autocrine/paracrine loop. (201). A recent report unravels a novel mechanism of GBM invasion via CD95 receptor-mediated of apoptosis-resistant cells through upregulation of MMP-2 and MMP-9 levels. (202).

In addition, several growth factors and cytokines, intratumoral hypoxia, and recently identified CD133-positive glioma stem cells can also stimulate VEGF expression (203-207), thereby inducing angiogenesis and invasion in gliomas. Recent results further support the concept that COX-2/PGE_2 levels are increased

in response to hypoxia, enhanced HIF-1α transcriptional activity and HIF-1α-mediated VEGF production via activation of Akt/ERK signaling pathway in colorectal cancers (171, 208). Based on these findings, anti-VEGF therapy may not be therapeutically successful since glioma cells can survive and propagate via host vessel co-option, glioma stem cell mediated angiogenesis or hypoxia-induced COX-2/PGE_2 signaling mediated through the Akt/ERK growth and survival pathways. Because stimuli such as intratumoral hypoxia, COX-2/PGE_2 and EP receptors co-stimulation signaling pathway (209, 210), *TP53* mutations (211) and genetic loss of *PTEN* (212, 213) can all trigger the angiogenic switch, combination strategies directed at the simultaneous inhibition of both angiogenic and invasive features of GBM progression may lead to improved patient survival outcomes.

1.5. Novel Therapeutic Approaches for Glioblastoma Treatment

1.6.1. Anti-VEGF-targeted Approaches

It is well established that tumor angiogenesis or neovascularization is primarily driven by the dominant pro-angiogenic factor VEGF signaling (214, 215). The best-characterized stimulus for increased VEGF expression is intratumoral hypoxia (216, 217), which either induces the activation of *VEGF*

gene transcription through the hypoxia-induced factor -1 (HIF-1) pathway (218) or up-regulation of VEGF mRNA stability mediated by the 3' untranslated region of the VEGF mRNA (219). GBM is one of the most vascularized tumors (220) and exhibits markedly increased expression of VEGF (221). In situ hybridization studies have shown that VEGF mRNA is highly localized to the surrounding pseudopalisading hypoxic and necrotic foci in GBM (222). VEGF expression is correlated with high tumor grade and poor prognosis in patients with brain tumors (223-225). In addition to hypoxia-induced VEGF activation, the increased VEGF activity in GBM can also be triggered by numerous growth factors and cytokines such as insulin-like growth factor (IGF)/IGFR (226, 227), EGF/EGFR via the commonly activated PI3K signaling pathway (228, 229), IL-6 (205) and tumor necrosis factor-α (TNF-α) (230)-mediated in part through the transcription factor Sp1. Furthermore, overexpression of Src or Ras can upregulate VEGF transcriptional activity (231). In addition to these mechanisms, two recent reports have shown that FoxM1B transcription factor (232) and HuR protein (233) are upregulated in GBM leading to increased VEGF expression which is mediated by an independent or dependent HIF-1α pathway, respectively. Taken together, these preclinical studies continue to suggest that VEGF may be an attractive therapeutic target for gliomas.

It is well-recognized that the VEGF signaling pathway is the cornerstone of the angiogenic signaling axis in GBM. Mounting evidence has demonstrated the efficacy of inhibition of the VEGF signaling cascade via blockade of the VEGF or VEGF receptor (VEGFR) in suppressing tumor growth and angiogenesis in

gliomas (234-237). Several anti-VEGF-targeted agents have been developed to target VEGF, VEGF receptors and tyrosine kinase inhibitors with selectivity for VEGF receptors (VEGFR-TKI). The most prominent agents approved by the FDA for clinical use are bevacizumab (Avastin; humanized anti-VEGF antibody) and sorafenib and sunitinib (anti-VEGFR-TKIs) (238-241). Several mechanisms of action for anti-VEGF-targeted therapy have been proposed. Studies in murine models have shown that targeting VEGF could induce endothelial cell apoptosis but rarely regression of established tumors (242, 243). Other studies demonstrated that the mechanisms of action with bevacizumab may be to suppress the mobilization and levels of circulating bone marrow-derived endothelial progenitor cells (EPC) thus preventing the incorporation rate of EPCs into the tumor vasculature (244). Most importantly, it has been shown that anti-VEGF-target therapy induces normalization of the tumor vascular bed (245, 246), leading to redistribution of blood flow and improvement of concomitant anti-neoplastic drug delivery to the tumors (247, 248). Preclinical evidence has shown that a number of anti-VEGFR-TKIs exhibit both anti-tumor and anti-angiogenic activities. These agents include sorafenib (Nexavar®; Onyx, CA USA) (249) and vandetanib (ZD6474; AstraZeneca, UK) (250). Unfortunately, many of these VEGFR-TKIs drugs induce off-target effects in addition to VEGFR inhibition owing to their broad-spectrum activity. Furthermore, two recent independent studies by Ebos *et al.* (251) and Paez-ribes *et al.* (252) report that blocking tumor angiogenesis with VEGF-targeted therapies not only inhibits primary tumor growth in mouse models but also can accelerate tumor cell spread and enhance metastases to distant

organs, leading to accelerated death when compared with control mice. These findings are in contrast to previous studies which demonstrated that anti-VEGF therapy inhibited primary tumor growth and reduced metastasis (253, 254). One plausible mechanism for this disparity may be the degree of intratumoral hypoxia, which is an important driving force for tumor growth and malignant progression (255-257). Perhaps, targeting the multitude of angiogenic factors (VEGF, IL-8, FGF-2, angiogenin etc.) or angiogenic mediators in the tumor microenvironment including stromal fibroblasts, infiltrating immune cells, the extracellular matrix components may help control the problem of tumor angiogenesis.

1.6.2. Combined Anti-EGFR with Multiple Targeted Kinase Approaches

An aberrant EGFR signal pathway is observed approximately in 40-50% of primary GBM (258, 259). *EGFR* gene, encodes for a transmembrane receptor tyrosine kinase that mediates in signaling involved in cell survival, angiogenesis, and invasion (260), represents an another attractive therapeutic target for gliomas. However, the first clinical trials with an inhibitor of EGFR, i.e. gefitinib (Iressa; ZD1839) in gliomas produced disappointing results (261, 262). It was proposed that the resistance to treatment was associated with the co-expression of normal PTEN function and the mutant EGFRvIII (263, 264). In addition, clinical studies in GBM with erlotinib (Tarceva®; OSI-774), a specific inhibitor of EGFR, alone

or with TMZ and radiation demonstrated antitumor activity with minimal effect on overall survival benefits (265, 266).

A promising approach to overcome resistance to anti-EGFR-targeted therapy may include the use of combination of drugs that target invasive and metastatic behaviour of the tumor cells together with anti-VEGF-targeted drugs. The multiple targeted kinase inhibitors such as AEE788 (Novartis) and vandetanib (anti-EGFR and anti-VEGFR-2) that have both anti-angiogenic and anti-invasion properties have been recently developed, and these drugs have shown encouraging results in murine glioma xenografts models (262, 267). In fact, a trial using the combination of bevacizumab and dasatinib (BMS-354825), a small-molecule inhibitor targeting the pro-invasive Src kinase (268), is entering clinical trials for recurrent malignant glioma, and the efficacy of this combined treatment will be apparent in due course. Clinical trials of vandetinib in combination with TMZ plus radiation (Drappatz, J. *et al. J of Clinical Oncol.* 27(15S) *Abstrast #203, 2009*) and bevacizumab and erlotinib for GBM patients are also underway (Sathornsumetee, S. *et al. J of Clinical Oncol. 26(15S) Abstrast #13008, 2008*).

Despite the extensive research effort in glioma cancer biology, patients subjected to the current standard therapy (i.e. resection, radiation therapy with concurrent and adjuvant TMZ according to the Stupp Protocol) or novel anti-VEGF and/or anti-EGFR therapies continue to have a dismal prognosis. Addition of TMZ to the protocol was increased the median survival rate from 12.1 (i.e. RT alone) to 14.6 months (269) and a 5 year survivals of 9.8 % (270). Fewer than

50% of patients respond to these treatments (241, 271, 272). Although the mechanisms of responsiveness in GBM remain unclear (273), the presence of unidentified compensatory pathways and heterogeneous molecular profile in GBM likely contribute to the limited efficacy of these therapies. Several potential mechanisms by which tumor cells evade anti-VEGF-targeted therapy have been postulated (274, 275). These include endothelial and tumor cell heterogeneity, redundancy of pro-angiogenic growth factors and anti-apoptotic survival factor such as VEGF within the tumor microenvironment, and co-option of existing host vessels. Emerging evidence indicates that the presence of a small population (~10%) of stem-cell like glioma cells within the glioma tumors which exhibit radio-chemotherapy resistance, are responsible for tumor initiation and recurrence (191, 276, 277). Another possible mechanism recently reported suggests that during the course of anti-VEGF treatment, vessel regression causes hypoxia which can further induce the recruitment of circulating endothelial progenitor cells (EPCs) towards to the tumor site and up-regulate pro-angiogenic factors such as SDF1α/CXCR4, all of which facilitate neovascularisation (278, 279).

Ultimately, there is an urgent need for the characterization and identification of signaling pathways involved in GBM tumor progression and invasion that can be therapeutically targeted. As glioblastoma cancer biology is complicated by the fact that these tumors display tremendous genomic and proteomic heterogeneity, successful treatment of GBM will likely depend on the utilization of a "cocktail" of agents that are individually tailored toward a unique genomic profile in GBM patients. In most neuropathological centres, GBM's are

classified on the basis of histological features. However, diagnostic evaluation is beginning to change with the advent of gene expression profiling, comprehensive genomic and signaling pathway and network characterization as well as glioma stem cells identification. In addition, more complete surgical resection (i.e. 95-98%) utilizing computerized neuro-navigational, fluorescent dyes and intraoperative MRI's has recently been shown to be associated with improved survival times (280). Neurobiologists continue to unravel previously unrecognized putative targets of cell proliferation, survival, angiogenesis, and invasion. These novel targets will foster new vistas for drug development strategy which hopefully will translate into improved therapeutic results.

1.7. Statement of the Hypothesis

Utilizing the aforementioned PGE_2 paradigm as a cancer research resource, this thesis project explores a novel COX-2/PGE_2 signaling pathway and molecular cross-talk with other oncogenic pathways involved in GBM cell proliferation and invasion. A series of three approaches, as outlined in **Figure 1-4**, addresses the central theme: **the characterization and identification of a novel COX-2/PGE_2 signaling in glioblastoma cells**. In each case, we have characterized the molecular biology of functional proteins and identified several key signaling molecules involved in modulation of glioma cell growth, angiogenesis and invasion. In the first cell-based screening approach, we attempt to validate the

new putative dual COX/LOX inhibitor compounds for their COX-2 and 5-LOX biological functions. In Chapter 2 we describe the pharmacological and molecular characterization of these dual COX/LOX inhibitors. We screened a large panel of 12 early passage primary cultures of human glioma cell lines (biopsy derived) and identified a significant number of cell lines whose COX-2 expression and PGE_2 production are elevated. Unexpectedly, we discovered that the putative dual inhibitors (compounds 13e, 13g and 13i) exhibited a lack of appreciable COX-2 and 5-LOX antagonistic function as reflected by cell-based screening assays. However, molecular analysis show that these compounds induced both a cell cycle G_0/G_1 phase block and apoptosis through a caspase-3 mediated proteolysis of β-catenin via cell-cell adherent pathway. Surprisingly, our results suggest that the apoptosis-inducing activity of these compounds is not associated with their COX/LOX activities. Thus, these diarylprop-2-yn-1-one moiety compounds may represent a lead compound for the development of a novel class of non-COX/LOX chemotherapeutic agents. Owing to the high concentration of these compounds required to induce apoptotic cell death, caution is advised regarding potential off-target effects.

Because of the retrospective evidence associated with COX-2 expression pattern in GBM, we hypothesized that COX-2-derived PGE_2 promotes glioblastoma cell growth and invasion. Utilizing molecular and pharmacological approaches, we demonstrate that glioblastoma cell proliferation and invasion depends on a novel PGE_2 mitogenic signaling via the EP4 receptor mediated through transactivation of the EGFR, thereby leading to activation of both the

ERK1/2 and PI3K signaling pathways. These data suggest that EP4 receptor could be a potential drug target. In Chapter 3 we report for the first time to our knowledge that PGE_2-induced glioblastoma cell growth and Matrigel invasion requires the EP4-mediated transactivation of the EGFR and thereafter activation of the ERK1/2 and PI3K signaling pathways.

Resistance to anti-VEGF-targeted therapy remains a clinical challenge for patients with glioblastoma, and the mechanism of resistance may be associated in part to intratumoral hypoxia caused by vessel regression during the course of anti-VEGF treatment. In Chapter 4 we investigate the influence of hypoxia on PGE_2 signaling in GBM cells utilizing a hypoxia mimetic environment (0.5-1% O_2). We show that PGE_2 stimulation enhances the HIF-1α transcriptional activity and VEGF induction via a molecular cross-talk between the EP4-mediated HIF-1α protein, pro-angiogenic cytokines and its downstream mediators such as ERK1/2 and Akt in glioblastoma cells. This study highlights the potential benefit of EP4 receptor antagonism in the abrogation of GBM proliferation and angiogenesis.

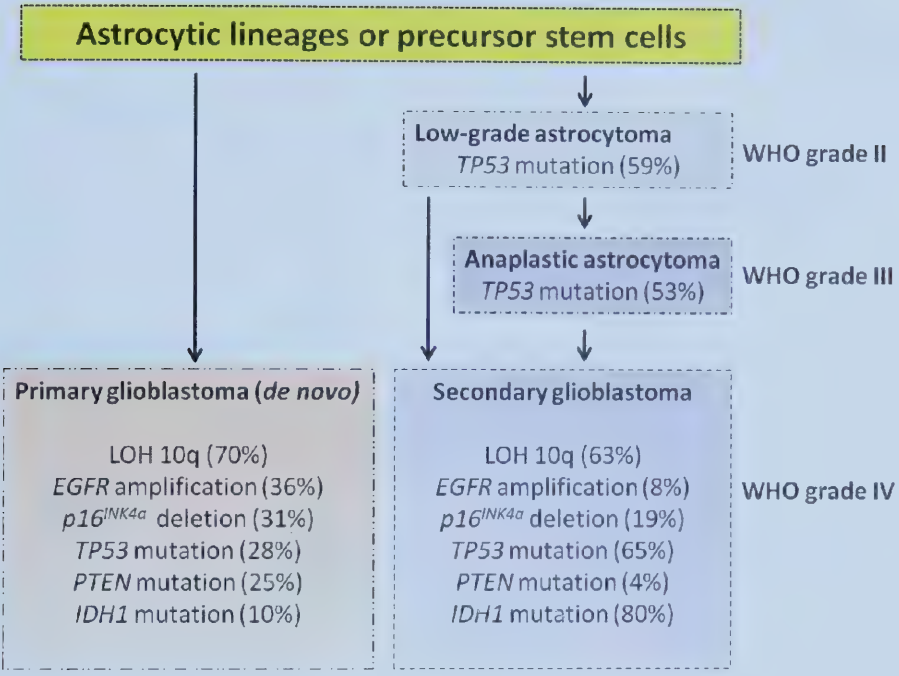


Figure 1-1. Genetic Pathways to Two glioblastoma Subtypes. Tumor progression to glioblastoma can develop without any evidence of prior clinical diseases (primary glioblastoma) or present at a low-grade astrocytoma or anaplastic astrocytoma over a period of 5-10 years of the initial diagnosis (secondary glioblastoma) (Adapted from Ohgaki et al. (5, 12) with minor modification).

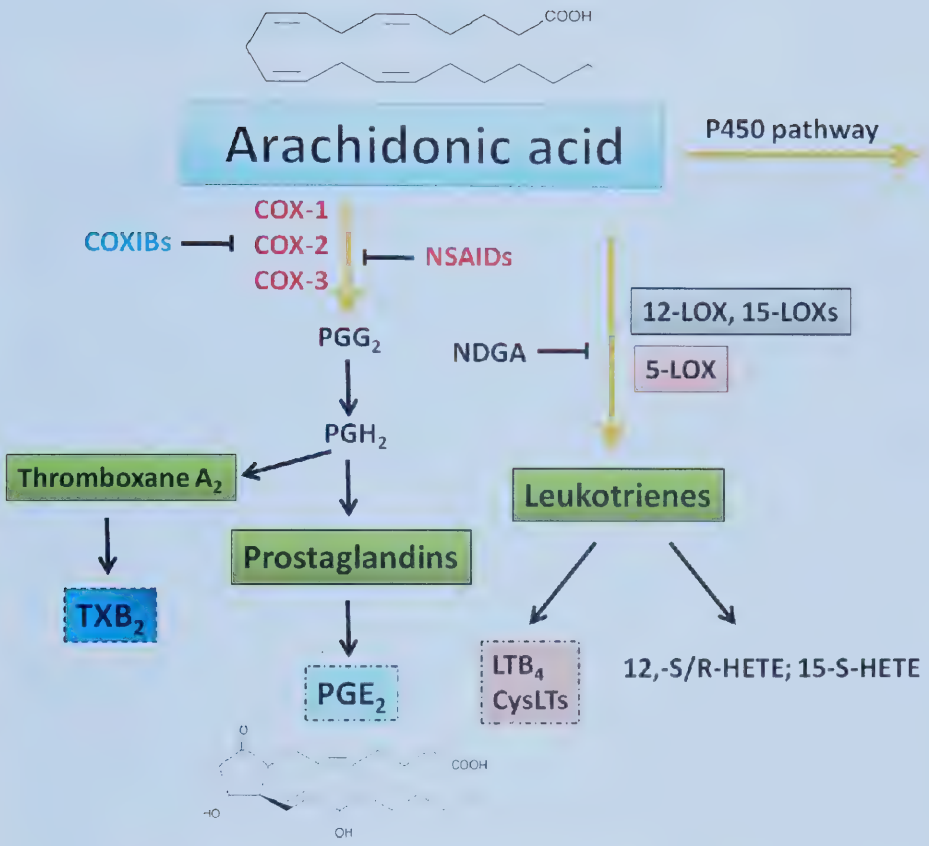


Figure 1-2. Overview of Arachidonic Acid (AA) Metabolism Pathways. The liberated AA can be metabolized via three major enzymatic pathways: cyclooxygenases (COXs), lipoxygenases (LOXs) and cytochrome p450 pathways. COXs catalyze the formation of prostaglandins and thromboxane A_2 from the liberated AA to form active metabolites of PGE_2 and thromboxane B_2 (TXB_2), respectively. LOXs catalyze the formation of leukotrienes (LTs) from the free AA to form active metabolites of LTB_4 and cysteinyl leukotrienes LTC_4, LTD_4 and LTE_4 (collectively called CysTLs). Cytochrome P450 involves the conversion of AA to active metabolites by NADPH-dependent oxidative reaction (not shown).

Table 1. Level of COX-2 expression in premalignant or malignant human cancer

(Modified from G. Gasparini et al. (74)).

Malignant /premalignant lesion	COX-2 expression (%)
Colorectal	80-90
Gastric	80
Oesophageal	70
Hepatocellular / liver cirrhosis	54/81
Pancreatic	67
Head and neck	80
Non-small-cell lung cancer	70
Breast / ductal carcinoma-in situ	40/60
Prostatic	83-93
Bladder	86
Cervix	43
Endometrial	37
Cutaneous basal cell	25
Cutaneous squamous cell	80
pPNET	100
Glioblastoma multiforme	71-74
Anaplastic astrocytoma / low grade	44/30

References available at http://image.thelancet.com/extras/03oncl205webfr.pdf

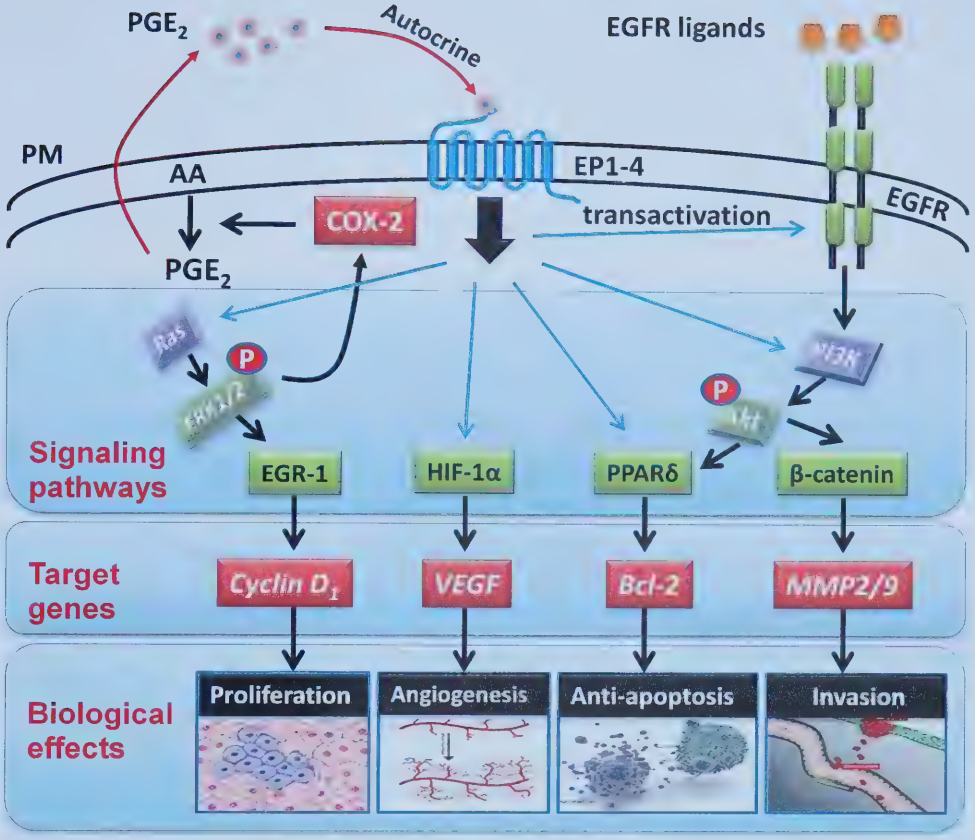


Figure 1-3. COX-2-derived PGE_2 Signaling Pathways and Associated Diverse Biological Activities. PGE_2 stimulation involves diverse biological effects in human tumorigenesis via all four prostanoid receptor subtypes EP1-4 through transactivation of EGFR and multiple downstream signaling pathways. Epidermal growth factor receptor (EGFR); phosphatidyinositol-3-kinase (PI3K); protein kinase B (Akt); peroxisome proliferator activated receptor δ (PPARδ); early growth response-1 (EGR-1); hypoxia-inducible factor-1 (HIF-1α); vascular epithelial growth factor (VEGF); matrix metalloproteinase (MMP); extracellular signal-regulated kinase 1 and 2 (ERK1/2); arachidonic acid (AA) and plasma membrane (281). Curved dark arrow depicts a positive feedback loop for upregulation of COX-2 expression. (Modified from Sanford D. Markowitz and Wang D (140, 141)).

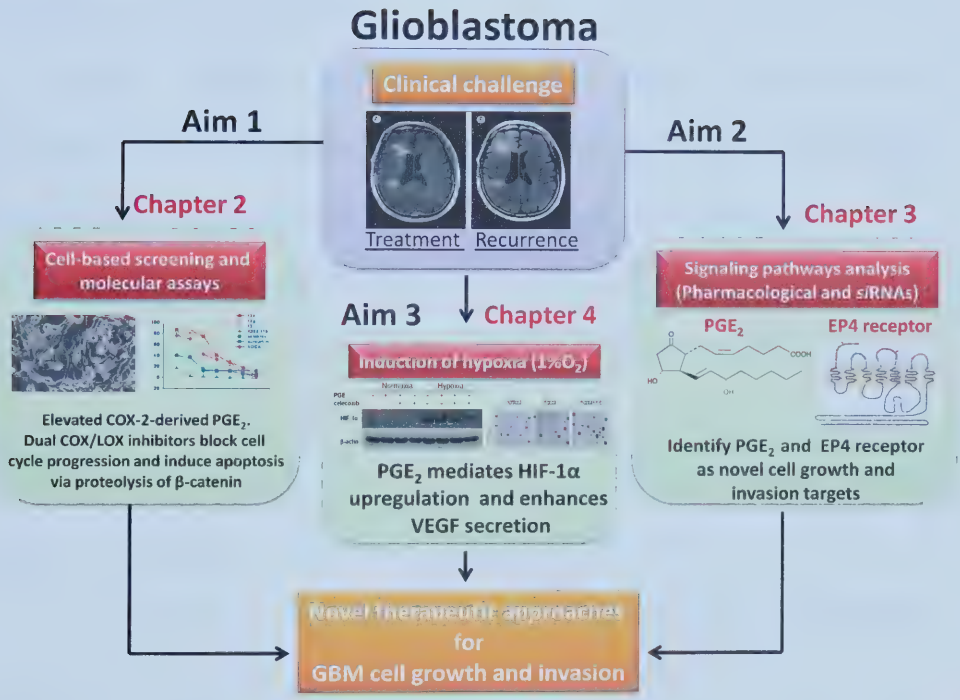

Glioblastoma
Clinical challenge
Treatment
Recurrence
Aim 1
Chapter 2
Cell-based screening and molecular assays
Elevated COX-2-derived PGE2. Dual COX/LOX inhibitors block cell cycle progression and induce apoptosis via proteolysis of β-catenin
Aim 2
Chapter 3
Signaling pathways analysis (Pharmacological and siRNAs)
PGE2
EP4 receptor
Identify PGE2 and EP4 receptor as novel cell growth and invasion targets
Aim 3
Chapter 4
Induction of hypoxia (1%O2)
PGE2 mediates HIF-1α upregulation and enhances VEGF secretion
Novel therapeutic approaches for GBM cell growth and invasion

Figure 1-4. Outline of the thesis

1.8. References

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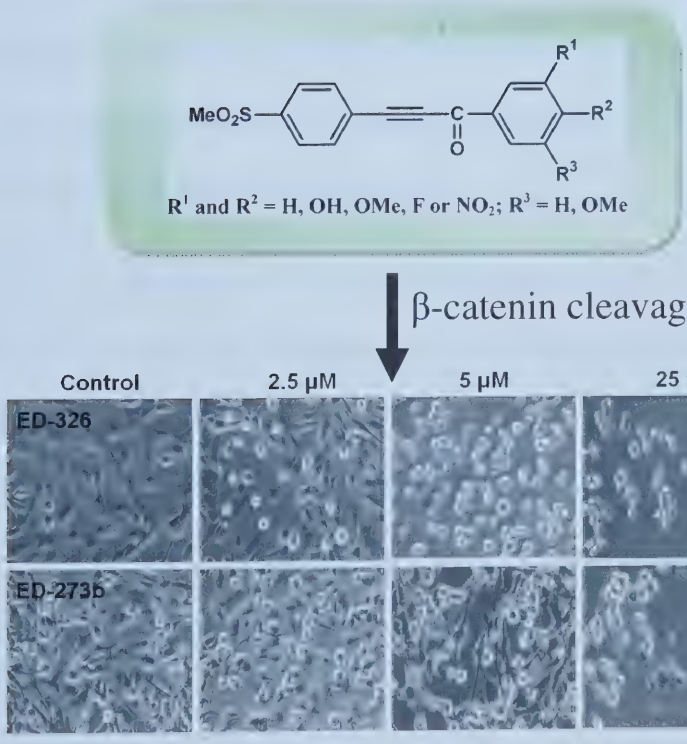
Chapter 2

Characterization of Dual COX/LOX Inhibitory 1,3-Diarylprop-2-yn-1-one Derivatives Reveals Caspase-Mediated Proteolysis of β-Catenin



2.1. Introduction

Substantial epidemiological and experimental evidence suggests that arachidonic acid (AA)-derived lipid mediators such as the prostaglandins (PGs), thromboxanes (TBXs), and leukotrienes (LTs) play pivotal roles in the evolution of inflammatory diseases as well as neoplastic initiation and progression (1, 2). Cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) are the key enzymes involved in AA metabolism. The AA metabolites, PGs and LTs, collectively termed eicosanoids (2) are well-characterized pathophysiologic mediators of inflammation and cancers (3, 4). Considerable evidence indicates that inhibition of COX-2 and/or 5-LOX activities, by non-steroidal anti-inflammatory drugs (NSAIDs), selective COX-2 or dual COX-2/5-LOX inhibitors, suppress tumor cell growth and angiogenesis, and induce apoptosis in a variety of cancer cell lines (5-8) including GBM (9-12). These results suggest that these compounds may have therapeutic potential against cancers.

COX-2 is overexpressed in many premalignant and malignant human cancers (13-18). In addition, overexpression of COX-2 and increased production of prostaglandin E_2 (PGE_2) are a frequent occurrence in human brain tumor tissues including malignant gliomas (19-25). Likewise, 5-LOX has been shown to be involved in the development of several cancers such as colon (26), oesophageal (27), and pancreatic cancer (28) and is linked to brain edema associated with cerebral ischemia (29). Furthermore, 5-LOX mRNA and protein are overexpressed in human astrocytomas and meningiomas (29, 30). As well, several

studies reported that COX-2 and 5-LOX are simultaneously coexpressed and upregulated in colon, lung breast and prostate cancer tissues and cell lines (6, 31, 32). Because these two enzymes share similarities of mechanisms of action and substrates, and a common convergent VEGF expression target (6, 32, 33), it is likely that inhibition of one arm such as the COX-2 pathway may activate the 5-LOX pathway with elevated levels of LTs. This "compensatory" pathway might explain the limited antitumorigenic effect of the selective COX-2 inhibitor celecoxib when utilized in physiological doses (< 30 - 40 μM) in colon cancer cells (34, 35). Therefore, targeting both enzymatic pathways using dual COX-2 and 5-LOX inhibitors may represent a more promising therapeutic approach by blocking both tumorigenic lipid mediators.

However, accumulating evidence suggests that the pharmacological effects of COX-2 activity inhibition and reduced tumor growth by NSAIDs or celecoxib are two separable structural features (36, 37). For example, NSAID derivatives, (sulindac sulfide and sulfone) (38, 39) or celecoxib derivatives (OSU-03012/OSU03013 and 2,5 dimethyl celecoxib-DMC) (40, 41), which lack COX-2 enzyme inhibition activities, still induce apoptosis in cancer cells. On the other hand, curcumin (a phenolic compound from the plant *Curcuma longa*) which exhibits dual COX-2 and LOX inhibitory activities (42), has been shown to reduce tumor growth in colon cancer cells via a non-COX-2 target through the induction of proteolytic cleavage of β-catenin pathway (43, 44).

β-catenin, originally identified as an essential component of cell-cell adhesion pathway, binds tightly to both the cytoplasmic domain of E-cadherin and

α-catenin to maintain the cadherin-catenin complex integrity on the cell membrane (45, 46). Genetic studies revealed that a putative second β-catenin pool in the cytoplasmic component of the Wnt/β-catenin signaling pathway mediates a signaling function via Wnt stimulation (47). In the presence of growth signals, β-catenin in the cytoplasm is released and binds to the T-cell factor (TCF) transcription factor, thereby initiating diverse transactivation of genes that are required for growth (48, 49). On the other hand, the β-catenin pool in the cadherin-catenin adhesion pathway promotes migration, invasion and metastatic properties via a cell-cell contact mechanism (48).

Of interest, it has been shown that PGE_2 utilizes Wtn/β-catenin signaling pathway to promote growth of colon cancer cells (50) and other studies showed that celecoxib, curcumin and the chemotherapeutic agent paclitaxel induced growth arrest and apoptosis via proteolysis of β-catenin (44, 51, 52). These studies highlight β-catenin as a potential chemopreventive target for cancer treatment and possibly GBM.

We previously reported the development of a series of new dual COX/LOX inhibitor compounds based on structure-activity relationship (SAR) acquired for the template of 1,3-diarylprop-2-yn-1-ones (53) (**Figure 2-2A**). These compounds have been tested *in vitro* using primary enzyme-based screening and in an animal model of inflammatory disease and showed a broad range of anti-COX-2 and anti-5-LOX enzymatic activities (53). In this study, we attempted to further validate the pharmacological inhibitory properties of these selected compounds in our established early explant primary glioblastoma cell lines using a secondary cell-

based screening model. In particular, we focused on the molecular mechanisms that underlie the growth inhibitory effects of these compounds on our primary GBM cell lines.

2.2 Materials and Methods

Drugs and Chemicals

A large panel of compounds was synthesized and developed previously based on the SAR data studies of a 1,3-diarylprop-2-yn-1-ones template. Some of the compounds designated 13-series compounds (13g, 13e and 13i) and K354-11b were tested *in vitro,* and showed enhanced potency with respect to inhibition of cyclooxygenase and lipoxygenase enzymatic activities. In the animal model, a significant anti-inflammatory and analgesic activity was noted (53-56). All the compounds were dissolved in dimethyl sulfoxide (DMSO) as 100X stock solution and frozen at -80°C in aliquots until further use. Celecoxib (Celebrex; 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzenesulfonamide was obtained from LKT laboratories, Inc. (St. Paul, MN). 2NTX-99 (4-methoxy-N1-[*trans*-4-4(nitrooxy)cyclohexyl)]-N3-(3-pyridinylmethyl)-1,3-benzenedicarboxa-mide, zileuton were purchased from Cayman Chemical (Ann Arbor, MI). Nordihydroguaiaretic acid (NDGA) and curcumin (diferuloylmethane) were purchased from Calbiochem (La Jolla, CA). Temozolomide (TMZ) was obtained from Schering, Canada Inc. (Pointe-Claire, QC, Canada). Etoposide (VP16) was

obtained from Novopharm Ltd. (Toronto, ON, Canada). Cisplatin (CDDP) was obtained from Faulding Inc. (Montreal, QC, Canada).

Primary Astrocytes and Glioblastoma Cell Cultures

Primary culture of normal human fetal astrocytes (NHA), a generous gift from Dr. V.Wee Yong (University of Calgary, Calgary, Alberta) was maintained in RPMI 1640 media in 75 cm^2 culture flask which was precoated with 1mg/mL of poly-L-lysine (Sigma-Aldrich) and used within the first 3-4 passages. Primary cultures of ED-273b, ED-326, ED-405, ED-407, ED-409, ED-418, ED-422, ED-439, ED-442, ED-460, ED-461 and ED-462 glioblastoma cell lines derived from surgical glioma tumor specimens were established as previously described (57). Long-term established glioma cell lines were obtained from Dr. Charles Hao (Emory University School of Medicine, Atlanta, GA). The human colon cancer cell lines HCT-116, DLD-1, $CaCo_2$ were a kind gift from Dr. Michael Weinfeld (Cross Cancer Institute, Edmonton, Alberta) and the prostate cancer PC-3 and breast cancer MCF-7 cell lines were obtained from Dr. Joan Turner (Cross Cancer Institute, Edmonton, Alberta). All glioblastoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) and F-12 nutrition mixture (Invitrogen, Carlsbad, CA); colon, breast, and prostate cancer cells were maintained in DMEM containing 10% fetal bovine serum, 100 units/mL penicillin, and 100 units/mL streptomycin at 37°C.

Measurement of PGE_2, Thromboxane B_2 and Cysteinyl Leukotrienes Productions

ED-326 and ED-273b primary cultures of GBM cells (5 x 10^5/well) were seeded onto 24-well plates overnight to attain 75-80% confluence. Cells were treated with various concentrations of the experimental and chemotherapeutic drugs for 24 h and the culture supernatants were collected and assayed for PGE_2 (an index for COX-2 activity), thromboxane B_2 (TXB_2) (an index for COX-1 activity) and cysteinyl leukotrienes (CysLTs) production using enzyme immunoassay (EIA) kits (Cayman Chemical, Arbor, MI) according to the manufacturer's instructions. CysLTs EIA detects a group of LT metabolites such as LTC_4, LTD_4 and LTE_4 and is used as a readout for 5-LOX activity. The samples were assessed in triplicate and measured with a Benchmark microplate reader Model 680 (Bio-Rad Laboratories, Hercules, CA). The final mean values were expressed as picograms per millilitre per 5 x 10^5 cells (pg/mL/cells).

Western Blot Analysis

Established primary and long-term cultures of glioblastoma cells were rinsed in PBS and then lysed in a commercial PhosphoSafe extraction buffer (Novagen, Gibbstown, NJ) containing 1mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor tablets (Roche Diagnostics, Laval, Quebec, Canada) for 30 min on ice and centrifuged at 14,000 rpm for 15 min at 4°C. In some experiments, cells were separated into Triton X-100 soluble and insoluble fractions using Lampugnani et al. protocol (58). In brief, cells were extracted with

lysis buffer [10 mM Tris-HCL, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1% NP-40, 1% Triton X-100 and protease inhibitor tablets (Roche Diagnostics)] for 30 min on ice and centrifuged at 14,000 rpm for 10 min at 4°C. Supernatant was harvested as the Triton X-100 soluble fraction; the pellet was further extracted in the same lysis buffer as described above but substituted with 1% SDS on ice for 20 min each (2X). After centrifugation, the supernatant was collected as a Triton X-100 insoluble fraction. Protein concentrations of the cell lysates were determined using bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as a standard curve. Fifty micrograms (50μg) of the proteins was resolved on SDS-PAGE and transferred to a 0.45μm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, and CA). The membrane was blocked with 5% non-fat dry milk in TTBS (20 mM Tris-HCL[pH7.5] + 150 mM NaCl + 0.5% Tween-20) buffer for 1 h and then incubated overnight at 4°C with the following primary antibodies: anti-COX-1 and anti-COX-2 from Cayman Chemicals (Ann Arbor, MI); anti-β-catenin and anti α-catenin from Sigma-Aldrich Canada Ltd.(Oakville, ON, Canada); anti-E-cadherin and anti-cyclin D1 from Calbiochem (La Jolla, CA); anti-5-LOX from BD Biosciences (Bedford, MA); anti-caspase-3 and anti-$p21^{wafl}$ from Cell Signaling Technology (Denvers, MA) and anti-ERK1/2 from Stressgen Bioreagents (Ann Arbor, MI). After three washes (10 min for each wash) with TTBS, the membrane was incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 45 min at room temperature and followed by

three washes with TTBS. The immunoreactive protein bands were visualized by Western Lighting® chemiluminescence reagent plus (PerkinElmer, Boston, MA).

Flow Cytometric Analysis

Cells, plated at a density of 4 x 10^6/dish, were either treated with vehicle (0.1% DMSO) or compounds 13e, 13g, 13i at increasing concentrations for 48 h. The nonadherent and adherent cells were collected and centrifuged at 1,000 x g for 5 min. The pellets were washed once in ice-cold PBS and resuspended in 1 mL PBS followed by rapidly admixing the 1 mL cells into 9 mL of 80% ethanol in a centrifuge tube on ice and stored at -20°C for several days. For the cell cycle phase distribution study, the cells were rinsed in PBS and resuspended in a freshly prepared 0.5 mL DNA staining solution containing 50 µg/mL propidium iodide, 0.2 mg/mL RNase A and 0.15% Triton X-100 for at least 1 h before analysis. The cellular DNA content was analysed by a Becton Dickinson FACS-Calibur (BD Biosciences, San Jose, CA) flow cytometer using CELLQuest standard protocol for cell cycle distribution with at least 10,000 cells per sample were counted for each analysis.

For determination of apoptosis, the cells were washed in PBS, and stained with Annexin-V-FLUOS staining kit (Roche Diagnostics, Laval, QC) according to manufacturer's instructions. After 15 min staining, the samples were analyzed immediately on a FACS-Calibur flow cytometer. Annexin-FITC fluorescence

intensity was measured in duplicate and expressed as percentage of Annexin-V positive cells of at least 10,000 total cell counted.

Cytotoxicity XTT Assay

Normal human fetal astrocytes, primary GBM cultures of ED-273b and ED-326 (5 x 10^3cells/well) were seeded in 96-well plates and incubated at 37°C overnight to attain 75-80% confluence. The cells were either treated with vehicle (0.1% DMSO) or increasing concentrations of compounds K354-11b, 13g, 13e, and 13i (1.56-100μM) for 48 h. In some cases, cells were pretreated with chemotherapeutic drugs TMZ (7.5-500 μM), VP-16 and CDDP (0.15-10 μM) in the presence of 2% FBS for 24 h, followed by 13e (2.5 and 5 μM) for another 48 h. Cell viability was assessed using proliferating kit II (XTT) (Roche Molecular Biochemicals, Laval, QC) assay according to manufacturer's protocol. In brief, 50 μL of XTT labelling mixture (5mL XTT labelling reagent with 0.1 mL electron labelling reagent) was added to each well and incubated for 4 h at 37°C. The absorbance of the samples containing the orange formazan dye was directly quantified with use of a Benchmark ELISA microplate reader Model 680 (Bio-Rad Laboratory, Mississauga, ON) measured at 492 nm against a reference wavelength of 650 nm. Each sample was done in triplicate. The percentage of cytotoxicity was calculated from the equation, % cytotoxicity = (1 – O.D. treatment / O.D. control) x 100%, whereas O.D denoted as optical density. Dose

response curves were plotted and concentrations of the drugs at which caused 50% growth inhibition (IC_{50}) were calculated by linear interpolation.

Apoptosis Assay

ED-326 and ED-273b primary glioblastoma cells and MCF-7 breast cancer cells (caspase-3 null cells) were plated at a density of 2 $x10^6$/dish and incubated at 37°C overnight. Culture dishes were treated with compounds 13e and 13g at increasing concentrations (20, 40 and 60 μM) or untreated (control) for 24 h. The dishes were then washed once in ice cold PBS, scraped in 1 mL PBS and centrifuged at low speed 300 x g for 10 min. The cells were lysed in PhosphoSafe extraction buffer containing 1mM PMSF and protease inhibitor tablets for 30 min on ice and centrifuged at 14,000 rpm for 15 min at 4°C. Protein concentrations of the cell lysates were determined using bicinchoninic acid (BCA) protein assay kit. The caspase-3 activity was quantified in the supernatant using a caspase-3 activity fluorimetric assay kit (Roche Diagnostics, Laval, QC) according to the manufacturer's instructions. The final products were measured on Thermo Labsystems Fluoroskan Ascent FL fluorescence microplate reader (VWR, Mississauge, ON) with excitation filter 355 nm and emission filter 485 nm.

Immunofluoresence and Confocal Microscopy

Cells were plated on poly-l-ornithine-coated 4-well chamber glass slides overnight and were washed once in PBS and quickly fixed in 1:1 ratio of methanol and acetone for 5 min, and subjected immunostaining with anti-β-catenin for 2 h and then followed by Alexa 488-conjugated secondary antibody (Invitrogen, Carlsbad, CA) for 45 min and counterstained in Hoechst 33258 dye (Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) at 2.5 μg/mL for 5 min. The chamber slides were rinsed in PBS and mounted with mounting media (90% glycerol in PBS) and visualized on a Carl Zeiss Laser Scanning confocal Microscope (LSM510) and analyzed using the LSM imaging browser and Adobe® Photoshop 7.

2.3. Results

Endogenous Expression of COX1/2 and 5-LOX in Primary Glioblastoma Cells

We first analyzed a large panel of glioblastoma cell lines for endogenous expression of cyclooxygenases (COX-1 and COX-2) and lipoxygenase (5-LOX) by immunoblotting using primary specific antibodies against COX-1, COX-2 and 5-LOX. As shown in **Figure 2-1**, COX-1 expression was detected in approximately 40% of the 14 cell lines tested, including U-87, ED-273b, ED-461, ED-460, and ED-405 cells. Interestingly, COX-2 expression was detected in more

than 50% of the cell lines tested. The highest levels of COX-2 protein expression was observed in ED-273b and ED-326 cells followed by ED-460, ED-405 and ED-418 cells, then LN-18, ED-442 cells and U-87, with ED-407 cells expressing the lowest levels of COX-2 protein. However, 5-LOX protein expression was detected with similar levels of expression in all the cell lines tested.

Effects of the Compounds on PGE_2, TXB_2 and CysTLs Productions

Having shown the differential COXs and 5-LOX protein expression profile in a broad spectrum of primary cultures of glioblastoma cell lines, we chose both ED-273b and ED-326 cell lines for our subsequent experiments largely because these two cell lines expressed both COX-2 and 5-LOX, and possessed a moderate growth rate. To determine the biological effects of the dual inhibitors on eicosanoid synthesis/release, cells were treated with appropriate concentrations of compounds K-354-11b, 13g, 13e and 13i for 24 h, and the culture supernatants were collected and measured for the production of TXB_2, PGE_2, or CysTLs as readouts for COX-1, COX-2, or 5-LOX activity, respectively. Treatment with K-354-11b moderately reduced the basal levels of PGE_2 and TXB_2 production in both cell lines only at the high concentration of 50 μM (**Figure 2-2B-E**). Similarly, compounds 13g and 13e treatment marginally reduced the basal levels of PGE_2 as well as TXB_2 in both cell lines, with the exception of 13i compound, which significantly reduced PGE_2 production only in ED-273 cells but not in ED-326 cells. 13i compound had no effect on both cell types for TXB_2 production

(**Figure 2-2B-E**). Notably, when compared with celecoxib and 2NTX-99 inhibitory control levels, these compounds only minimally inhibited the levels of COX-1 and COX-2 activities. In contrast, treatment with these compounds did not affect the CysTLs expression levels in both cell lines (**Figure 2-2F** and **G**). These data indicate that these compounds, in general, lack appreciable COX-1 and COX-2 activities as well as 5-LOX activity.

Cytotoxic Effects on Cell Growth

Next, we investigated the effects of compounds K354-11b, 13g, 13e, and 13i on the growth of normal human astrocytes and the two selected primary glioblastoma cell lines using a cytotoxicity XTT assay. Cells stimulated with the compounds of various concentrations, ranging from 1.56 - 100 μM, for 48 h, resulted in a dose-dependent cell growth inhibition (**Figure 2-3A**), which varied among the compounds and the cell lines. Notably, the estimated IC_{50} values for the compounds 13i and 13e were lower in both glioblastoma cell lines than that in normal human astrocytes, suggesting that these compounds have a greater cytotoxic potency for tumor cells when compared to normal astrocytes. Interestingly, there was little or no cytotoxic activity for K354-11b (11b) in all the three cell lines tested. To further demonstrate that the growth-cytotoxic effect was not restricted to these two cell lines, we further evaluated compounds 13e and 13i against a number of long-term established glioblastoma cell lines along with other primary glioma patient-derived cell lines as well as the normal human

astrocytes. As shown in **Figure 2-3B**, the compound 13e IC_{50} values ranged from 3 to 22 μM and 15 to 60 μM in primary and long-term glioblastoma cell lines, respectively. The compound 13i IC_{50} values ranged from 8 to 25 μM and 27 to 60 μM in primary and long-term glioblastoma cell lines, respectively. For normal human astrocytes , the IC_{50} values for compounds 13e and 13i were 28 μM and 60 μM, respectively. Taken together, these data indicate that compounds 13e followed by 13i exert greater growth-inhibitory effects in primary cultures of glioblastoma cell lines than that in the long-term established glioma cell lines as well as normal human astrocytes.

The Additive Effect of Compound 13e and Chemotherapeutic Drugs on Cell Growth

To examine whether the most potent compound 13e had an additive effect on cell growth in combination with chemotherapeutic drugs, ED-326 and ED-273b cells were pretreated with various concentrations of TMZ, VP16 or CDDP alone for 24 h or in combination with 2.5 μM or 5 μM of compound 13e for another 48 h. As shown in **Figure 2-3C**, chemotherapeutic drugs TMZ, VP16 and CDDP treatment alone dose-dependently inhibited cell growth with greater potency in ED-326 than that of ED-237b cells. Interestingly, when cells were pretreated with the chemodrugs for 24 h followed by addition of the compound 13e, there was an additively cytotoxic effect against ED-326 by a factor of 2-3 as compared with individual treatment. This enhanced effect was not seen with a

lower dose of compound 13e (2.5 μM). Similarly, a weak additive effect was observed at the 2.5 μM dose for ED-273b cells, whereas a marked additive effect was seen at the higher dose (5.0 μM) by addition of the compound 13e.

Effects of the 13-series-Compounds on Apoptosis and Cell Cycle Distribution

To determine whether the previously described growth-inhibitory effect of the compounds was associated with induction of apoptosis or cell cycle arrest, cells were treated with compounds 13e, 13g, and 13i at increasing concentrations for 24 h and the extent of apoptosis was assessed by flow cytometry analysis. Treatment with the compounds resulted in a significant increase in the percentage of apoptotic cells in a dose-dependent manner as determined by Annexin-V binding assay (**Figure 2-4A**). As apoptosis often occurs as a consequence of a cell cycle block, we performed cell cycle analysis and determined the percentage of cells based on propidium iodide stained DNA content by flow cytometry analysis. Treatment of cells with compounds 13e, 13g and 13i at a concentration of 40 μM caused an increase in the percentage of the G_0/G_1 phase (population of cells in cell cycle arrest) and the percentage of the sub-G_1 phase (population of cells in apoptosis), compared with vehicle treatment alone (**Figure 2-4B**) in ED-326 cells. Likewise, the compounds treatment showed a similar level increase in G_0/G_1 but along with G_2/M phase and with a lesser extent of apoptosis in ED-273b cells.

It is well established that the progression through the cell cycle phases is driven by cyclins and cyclin-dependent kinases (Cdks) such as $p21^{Waf1}$ and

$p27^{Kip1}$ (59, 60). We further investigated whether these compounds induce G_1 phase growth arrest by examining the cyclin D1 kinase activity (a marker for the G_1 phase arrest). Western blot analysis show that a marked dose-dependent decrease of cyclin D1 expression was observed in the cells treated with compounds 13g and 13e (**Figure 2-4C**) whereas the expression of $p21^{Waf1}$ was not altered by the compounds treatment (data not shown).

Effects of the 13-series Compounds on the Subcellular β-catenin Trafficking

Recent studies have shown that celecoxib and/or curcumin (a COX-2/LOXs inhibitor) induces growth arrest and apoptosis through a COX-2-independent pathway via alteration of the β-catenin trafficking and induction of β-catenin degradation in colon carcinoma cells (44, 51). First, we investigated endogenous level of the β-catenin subcellular distribution in GBM, normal human astrocytes and colon cancer cells by immunofluorescence staining. Confocal microscopy analysis showed that the subcellular β-catenin distribution was localized both in the cytoplasm with a punctuate staining pattern (arrows) as well as within the cell membrane with less intensive staining in primary and long-term cultures of GBM cells (**Figure 2-5A**). In contrast, the β-catenin distribution was observed mainly within the cell membrane of HCT-116, DLD-1 and $CaCo_2$ colon cancer cells, with the low expression levels observed in the cytoplasm, which are consistent with previously published data (51, 61). Likewise, a similar pattern of staining of the β-catenin was observed in normal human astrocytes as observed in colon cancer

cells (**Figure 2-5A**). These results suggest that the constitutive β-catenin subcellular distribution varies with tumor cell types as well as normal astrocytes.

It is known that β-catenin exists in the cell membrane (cell-cell adhesion function) and within the cytoplasm and nucleus. Next, we examined β-catenin trafficking at the subcellular level in ED-326 and ED-273b glioblastoma cells. After treatment with compound 13e for 4 h, GBM cells gradually became morphologically rounded up and lost their cell-cell contacts with neighbourhood cells in a dose-dependent manner (**Figure 2-5B**). To explore further at the molecular level, we treated these cells with compounds 13g and 13e at concentrations ranging from 0 to 80 μM for 24 h and evaluated their effects by western blotting. As shown in **Figure 2-5C**, induction of the β-catenin cleavage occurred in a dose-dependent fashion starting at approximately 60 μM in both cell lines. No cleavage was witnessed in γ-catenin. Cells treated with 60 μM 13g and 13e for periods of 0 to 24 h, similar time-dependent effects were seen in degradation of the β-catenin (**Figure 2-5D**). In addition, a lack of E-cadherin expression was observed in both cell lines (**Figure 2-5C**). However, no β-catenin cleavage was found in cells treated at concentrations of 0 to 100 μM with K-354-11b or celecoxib (data not shown). To further demonstrate that β-catenin cleavage was not cell-type-restricted, we treated human prostate PC-3 and colon HCT-116 cancer cells with various concentrations of compounds 13g, 13e, 13i, celecoxib (CXB) and curcumin, as indicated, for 24 h and compared their respective biological effects on β-catenin trafficking. Induction of β-catenin cleavage by the compounds 13g, 13e and 13i was observed in both cell types with much greater

effect on HCT-116 than that of PC-3 cells whereas no β-catenin degradation was found in these cells when treated with celecoxib or curcumin at concentrations up to 100 or 50 μM, respectively (**Figure 2-5E**). Taken together, our results demonstrate that compounds 13g, 13e as well as 13i (data not shown) induce β-catenin proteolytic cleavages in a wide spectrum of cancer cells.

Effects of the 13-series Compounds on Caspase-3-mediated β-catenin Degradation

Caspase activation is required for many of the morphologic and biochemical changes involved in apoptosis. Evidence suggests that caspase-3 is the primary enzyme responsible for proteolytic cleavage of β-catenin during apoptosis induced by CDDP and staurosporine (62, 63). To investigate the mechanism(s) by which β-catenin is degraded, we treated glioblastoma ED-273b and ED-326 cells, and human breast cancer MCF-7 cells (which lacks of caspase 3 activity as a control)(64) with 13g and 13e at selected concentrations of 20 to 60 μM for 24 h and measured the induction of caspase-3 activity using caspase-3 activity fluorometric assay. As shown in **Figure 2-6A**, caspase-3 activity was increased in a dose-dependent fashion after the compound treatment in both ED-273b and ED-326 cells. In contrast, caspase-3 activation was not observed in the caspase-3 null (i.e. *CASP-3* gene deleted) MCF-7 cells. Western blot analysis further confirmed that compound 13g and 13e treatment resulted in a dose-dependent cleavage of pro-caspase-3 in ED-326 glioblastoma cells (**Figure 2-6B**).

Notably, these results were correlated with induction of growth arrest as well as cleavage of β-catenin, suggesting that 13g/13e-induced apoptosis was mediated through a caspase-3 dependent β-catenin proteolytic cleavage.

To gain further insight into what subcellular compartment the β-catenin cleavage took place, we chemically isolated the membrane and cytosolic fractions from the compound-treated cells using Triton X-100 detergent according to the established protocol (58) and thereafter subjected these fractions to western blot analysis. Biochemical studies demonstrate that the proteolytic fragments of β-catenin occurring in the 13g, 13e and 13i treated cells were detected predominantly in the membrane fractions and observed less appreciably in the soluble fractions (**Figure 2-6C**), suggesting that β-catenin degradation takes place predominantly within the cell membranes and to a lesser extent in the cytoplasm. The importance of this finding requires further investigation.

2.4. Discussion

Substantial evidence has indicated that COX-2 and 5-LOX are co-expressed and upregulated in various types of human cancers and that their metabolites, the eicosanoids, stimulate tumor cell growth and promote neoangiogenesis (6, 31). Inhibition of these metabolizing enzyme activities suppressed growth and induced apoptosis in cancer cell lines and preclinical models (8, 31, 65, 66). Hence, these enzymes or their metabolites (eicosanoids) may provide promising targets for

therapeutic drug development. In keeping with this assumption, western blot results show that the majority of primary glioblastoma cells co-expressed the COX-2 protein and the universally expressed 5-LOX protein, supporting the concept that these enzymes and/or their metabolites may be involved in glioblastoma tumorigenesis.

In addition, recent evidence indicates that the inhibition of COX-2 or 5-LOX enzymatic activity by NSAIDs, in particular, celecoxib and 5-LOX inhibitor or in combination with celecoxib has been associated with reduced tumor growth and apoptosis, suppression of angiogenesis, and invasion (67-72); however, the underlying molecular mechanism(s) by which COX2/5-LOX inhibitors exert apoptotic cell death in a variety of cancer cells has remained the focus of intensive investigations. In fact, there is increasing evidence to show that many selective COX-2 inhibitors including NSAIDs also potently induce apoptosis via COX-2-independent mechanisms (43, 73, 74). These studies suggest inhibition of COX-2 activity is not a mandatory requirement for apoptotic activity. Indeed, SAR analysis via structural modification of celecoxib derivatives reveals that the structural requirements for COX-2 inhibitory activity are distinct from those for apoptosis induction (37, 41). Based on this structure-activity analysis and molecular modeling, several celecoxib derivatives, which lack COX-2-inhibitory function, have been established including 2,5-dimethyl-celecoxib (DMC) and OSU-03012 or OSU03013. These compounds have been shown to exert the antitumor effects via several non-COX-2 targets such as survivin and cyclins A

and B (75, 76) as well as 3-phosphoinositide-dependent kinase-1 activity (PDK-1) (40).

Our previous SAR studies provide the first measurable evidence that dual COX-2/5-LOX inhibitors could be developed by utilizing structural pharmacophore modifications (53, 56). Compounds depicting the 1,3-diphenylprop-2-yn-1-one structure have been shown to possess both anti-COX-2 and anti-5-LOX enzymatic activities *in vitro* and anti-inflammatory and analgesic activities in the animal model (53, 56). In the present study, we further evaluate the biological effects of these newly developed dual COX-2 and 5-LOX inhibitors in primary cultures of human glioblastoma cell lines. By measuring the eicosanoid synthesis/release in the cell culture supernatants after compound treatment at various doses, we are able to establish dose-response curves and predict the biological activity of these compounds. Although ED-326 and ED-273b glioblastoma cells secrete measurable amounts of PGE_2, TXB_2, and CysLTs, our results indicate that the 13-series compounds exhibit low anti- COX-1/COX-2 and 5-LOX activities when compared with the positive control agents such as celecoxib (selective COX-2 inhibitor) and curcumin (dual COX1/COX-2 and 5-LOX inhibitor). This new information contrasts to our previous published data (53). The reason for this discrepancy remains unclear and requires further investigation. However, it is possible that type-specific COX/LOX enzyme differences may play a role, in addition to the difference in eicosanoid sources used i.e. human PGE_2, TXB_2 and CysLTs in this study versus ovine $PGF_{2\alpha}$ and soybean LOX (*in vitro* enzymatic screening assay) used in our previous study.

Despite the limited anti-COX and LOX activities, the cytotoxic studies provide solid evidence that the compounds 13e, 13g and 13i except K-354-11b suppress tumor cell proliferation in a dose-dependent fashion with greater potency against glioblastoma cells than that of normal human astrocytes. This is demonstrated by the IC_{50} (5-22 μM) values (concentration for 50% inhibition) in GBM cells when compared to the IC_{50} (26-50μM) values in NHA. In addition, the cytotoxic effects of the compounds 13e and 13i on a panel of GBM cell lines reveal that these novel 13-series compounds are significantly more potent in inducing cytotoxicity when compared to celecoxib or curcumin. Notably, the compound 13e additively and dose-dependently augments the growth inhibitory effects of the chemotherapeutic drugs TMZ, VP16 and CCDP in primary GBM cells, suggesting that the addition of these compounds (i.e. 13e) could be utilized as an adjuvant therapy against GBM growth.

Furthermore, our data show that the 13-series compounds inhibited cell proliferation via G_0/G_1 phase cell cycle arrest which appears to be mediated through a decrease of the expression of cyclin D1 protein. The significance of G_2/M block observed in ED-273b cells is not known and requires further investigation. It is well-recognized that inhibition of D-type cyclins can cause G_0/G_1 growth arrest and that increased p21 also causes G_0/G_1 arrest via inhibiting phosphorylation of retinoblastoma (Rb) by cyclin-dependent kinases 4/6 at the G_1/S transition (59, 77). However, our studies show that these compounds did not cause alternations of the expression of cell cycle regulatory protein p21. Notably, the growth-inhibitory effects of the 13-series compounds were associated with

induction of apoptosis in primary cultures of glioblastoma cell lines. It is known that prolonged cytostatic effects can triggers apoptosis and cell death (78). Although our results showed both the cytostatic (cell cycle arrest) and cytotoxic (Annexin-V and XTT assays) effects of the 13-series compounds, it remains to be clarified whether the cytotoxic effect is totally or partially mediated by tumor cell cytostasis without direct cytotoxic effects. Nevertheless, the ability of these compounds to inhibit cell growth and induce apoptosis, the two most important mechanisms underlying the anti-tumor effect of NSAIDs, selective COX-2 or dual COX/LOX inhibitors, has prompted us to investigate the biological impact of the 13-series compounds on GBM cell function at the molecular level and whether these compounds can serve as potential chemotherapeutic drugs against this devastating disease.

In this study, we report that the 13-series compounds which lack appreciable COX and LOX activity potently induce apoptosis via proteolytic cleavage of β-catenin in primary glioblastoma cells. β-catenin is a component of cell-cell adherens junctions which possesses both structural and signaling function (79). The induced cleavage of β-catenin appears to be specific. We show that treatment with compounds 13g and 13e trigger a dose-dependent and time dependent cleavage of β-catenin but not α-catenin (another component of the cell-cell contact complex) in primary glioblastoma cells. Failure to show the involvement of E-cadherin cleavage in these cells could be due to a mutation or downregulated E-cadherin expression which is often observed in various types of cancers (80) or due to the fact that the antibody that we used was not specific

enough. Second, we demonstrate that the 13-series compound-treated cells changed morphologically by becoming "rounded up" in appearance and lost cell-cell contacts in 2-3 h, suggesting that this effect is associated with the cadherin cell-cell adhesion mediated by the β-catenin/E-cadherin complex (81). In addition, the β-catenin cleavage-induced by the 13-series compounds was not cell-type restricted to GBM but was also seen in prostate cancer PC-3 and colon cancer HCT-116 cells. Interestingly, even at the concentrations of 50-100 μM, celecoxib and curcumin failed to induce β-catenin cleavages in PC-3 and HCT-116 cancer cells. Our results are supported by other studies which also demonstrated the cleavage of β-catenin and γ-catenin in apoptotic human embryo retinoblasts (82), human 3T3 fibroblasts (62), and in apoptotic human non-small lung cancers (52) after treatment with the chemotherapeutic agents CDDP and paclitaxel. Although we did not perform experiments to study the γ-catenin cleavage-effect of these compounds, we would expect that γ-catenin, like β-catenin, is also degraded during the compound-induced apoptosis. Nevertheless, our data show that apoptosis-inducing activity induced by these 13-series compounds mediates the cleavages of β-catenin to dismantle the cell-cell adhesion complex.

Previous studies by others have demonstrated that the role of caspase-3-mediated proteolysis of β-catenin is linked with apoptosis in various types of cancers (52, 63, 82, 83) and that degradation of β-catenin protein can cause disruption in the cell-cell adhesion pathway, leading to apoptosis (62, 83). In this study, we demonstrate that the activity of caspase-3 is increased after compounds 13e and 13g treatment in ED-326 and ED-273b glioblastoma cells but not in

breast cancer MCF-7 cells, which are deficient in caspase-3 expression (64, 84, 85). Our results also demonstrate that the concentrations required inducing caspase-3 cleavage correlate with the dose required for the cleavage of β-catenin in glioblastoma ED-326 cells. These findings are in agreement with the studies by others demonstrating that β-catenin can be directly cleaved by caspase-3 in celecoxib, paclitaxel and curcumin induced apoptosis in colon and lung cancer cells (44, 51, 52). Further studies are required to demonstrate whether β-catenin is a true substrate of caspase-3 in GBM cells by utilizing an *in vitro* translational study with purified caspase-3 and that decreased levels of β-catenin may increase sensitivity of apoptosis in GBM cells by use of siRNA to silence β-catenin protein expression.

In addition to its intercellular membrane-bound adhesion function, β-catenin also participates in transcriptional function by binding with TCF/LEF in the nucleus (86, 87). Although still debated, the existence of two cellular pools of β-catenin, suggests that these pools are associated with adhesion and transcription (88). The adhesive pool is regulated by tyrosine phosphorylation (89, 90) whereas the monomeric cytoplasmic pool is regulated by Wnt signaling to promote stabilization of β-catenin within the cytoplasm and the nucleus (91, 92). We investigate the biological effects of these compounds with respect to the subcellular localization of β-catenin by confocal microscopy and biochemical studies followed by western blotting analysis. Interestingly, we found that constitutive β-catenin staining was predominantly restricted within the cytoplasm as punctuate structures and less intensive staining within the cell membranes in

primary glioblastoma and established GBM cell lines. This pattern of staining was not altered in normal human astrocytes or colon cancer cell lines where the β-catenin staining was only observed within the cell membranes. These findings are further supported by a study showing the intracellular localization of β-catenin staining in GBM is different from that of glial cells within normal brain tissues (93). Furthermore, our biochemical studies demonstrate that the β-catenin cleavages were detected mainly in the detergent-insoluble fractions in both ED-326 and ED-273b glioblastoma cells. These data suggest that the proteolytic cleavages of β-catenin takes place within the membranes and/or nuclear structures. Although there is no direct evidence to show an alternative effect of these compounds on the transcriptional activity of β-catenin/TCF complex, further investigation is needed to provide a further insight into the apoptosis-inducing activity of these novel 13-series compounds by manipulating the levels of β-catenin in glioblastoma cells.

In conclusion, we provide evidence that the 13-series compounds treatment decreases growth of early passage cultures of glioblastoma and induces growth arrest mediated through Go/G_1 phase arrest. Our results indicate that the proteolysis of β-catenin induced by the compounds appears to be partially a consequence of caspase-3 activation, rather than a cause of apoptosis. The compound treatment causes an increase of caspase-3 activity, resulting cleavages of β-catenin to dismantle cell-cell adhesion, which may lead to apoptosis. The present findings suggest that proteolysis cleavage of β-catenin is not the mechanism by which the 13-series compounds induce apoptosis.

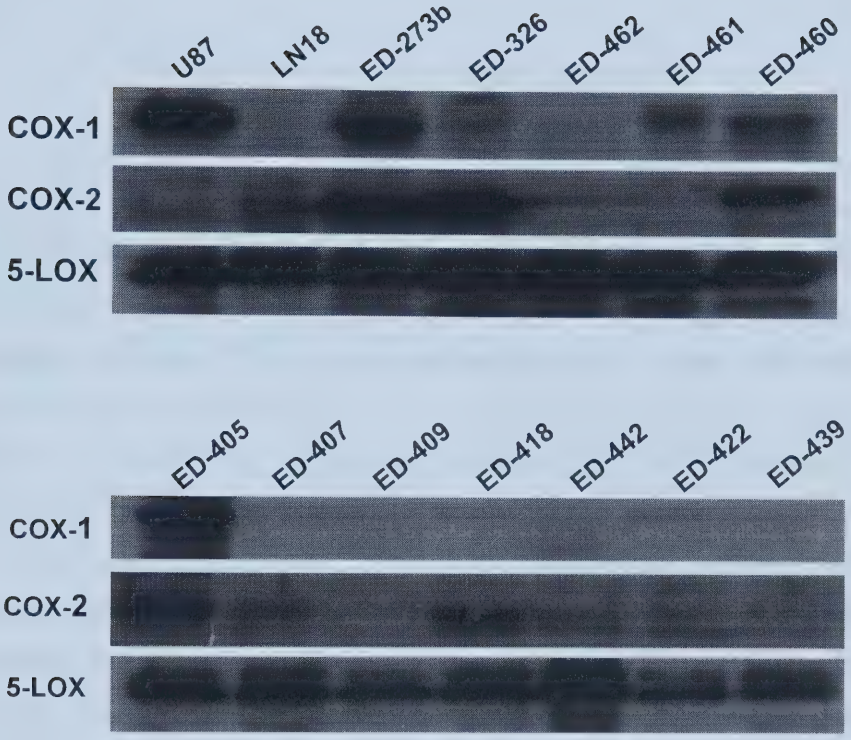
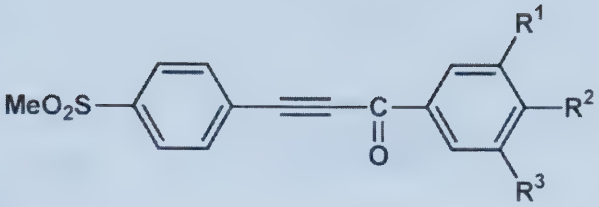


Figure 2-1. Endogenous COX-1/COX-2 and 5-LOX expression in human glioblastoma cell lines. Immunoblot analysis of cell lysates (50 μg) derived from the short-term cultured glioblastoma cell lines of ED-273b, ED-326, ED-462, ED-461, ED-460, ED-405, ED-407, ED-409, ED-418, ED-442, ED-422 and ED-439 and the long-term cultured glioblastoma cell lines of U-87 and LN-18 were separated onto 10% SDS-PAGE and electroblotted onto a nitrocellulose membrane. The membranes were probed with anti-COX-1, anti-COX-2 and anti-5-LOX antibodies.

Figure 2-2. Effects of compounds on COX-1, COX-2 and 5-LOX activities of primary cultures human glioblastoma cell lines. **A,** chemical structure of 1,3-diarylprop-2-yn-1-ones moiety compounds (k-354-11b, 13g, 13e and 13i) possessing a *p*-SO_2Me COX-2 pharmacophore on the C-3 phenyl ring. Primary ED-326 (**B, D, F**) and ED-273b (**C, E, G)** glioblastoma cells were untreated (control) or treated with celecoxib, curcumin, 2-NTX-99, zileuton, and compounds k-354-11b, 13g, 13e, and 13i at selected concentrations for 24 h. The spent culture supernatants were collected for determination of the levels of eicosanoid release affected by the inhibitor treatment. PGE_2 levels (**B and C**), marked for COX-2 activity; TXB_2 levels (**D and E**), marked for COX-1 activity; and CysLT levels (**F and G**), marked for 5-LOX activity were measured using enzyme immunoassays kit according to the manufacturer's protocols. Celecoxib served as a positive control for COX-2 activity; 2-NTX-99 served as a positive control for COX-1 activity; and curcumin or zileuton served as a positive control for 5-LOX activity. Columns shown were normalized to cell count and plotted as the average ± SEM of three independent experiments.

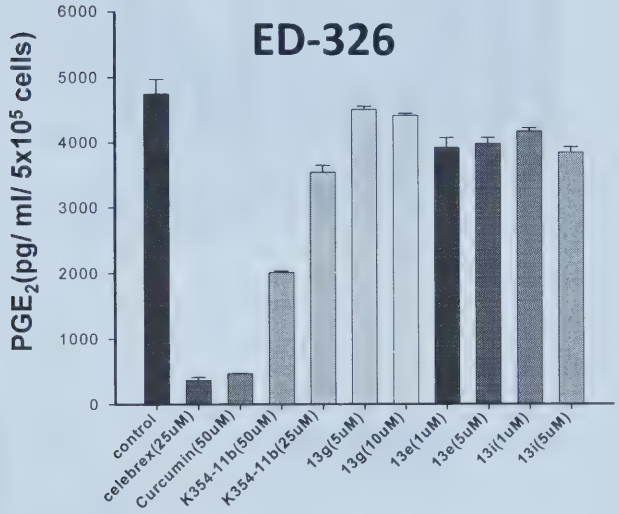
Figure 2-2.

A



	R^1	R^2	R^3
K-354-11b	H	Me	H
13e	H	*i*-Pr	H
13g	H	CN	H
13i	F	F	H

B



C

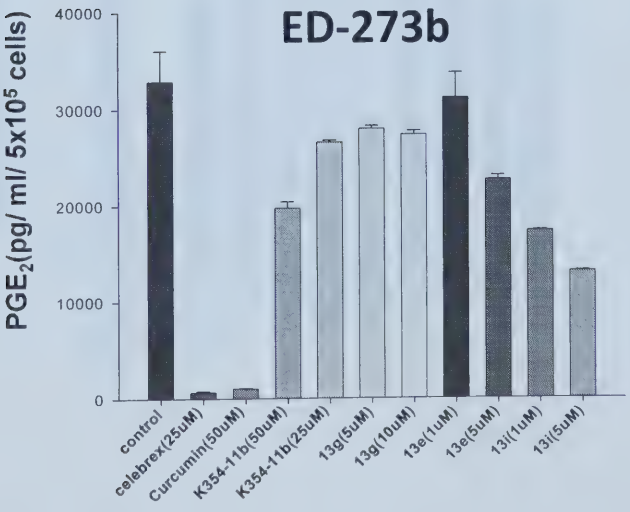
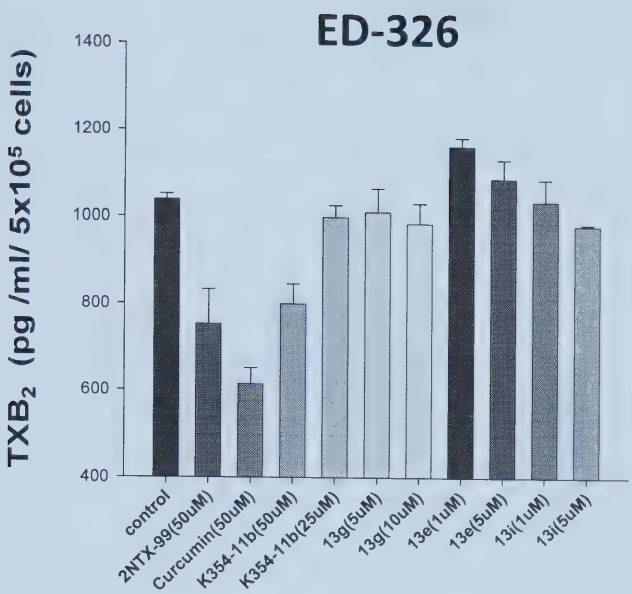


Figure 2-2.

D


ED-326
TXB_2 (pg /ml/ 5x10^5 cells)
1400
1200
1000
800
600
400
control
2NTX-99(50uM)
Curcumin(50uM)
K354-11b(50uM)
K354-11b(25uM)
13g(5uM)
13g(10uM)
13e(1uM)
13e(5uM)
13i(1uM)
13i(5uM)

E

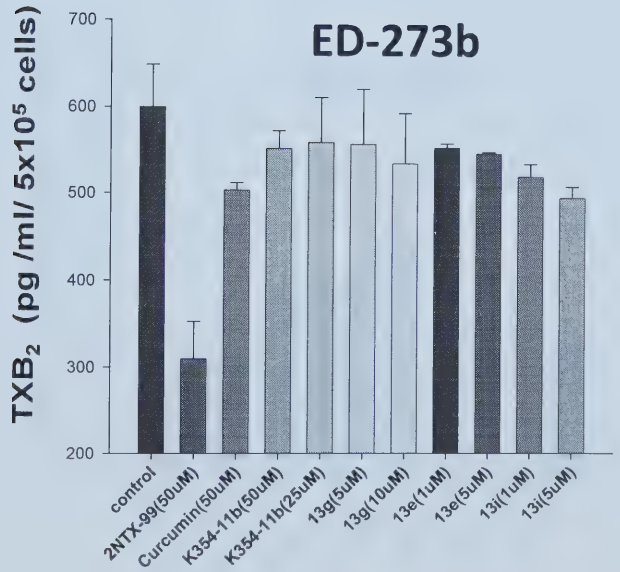
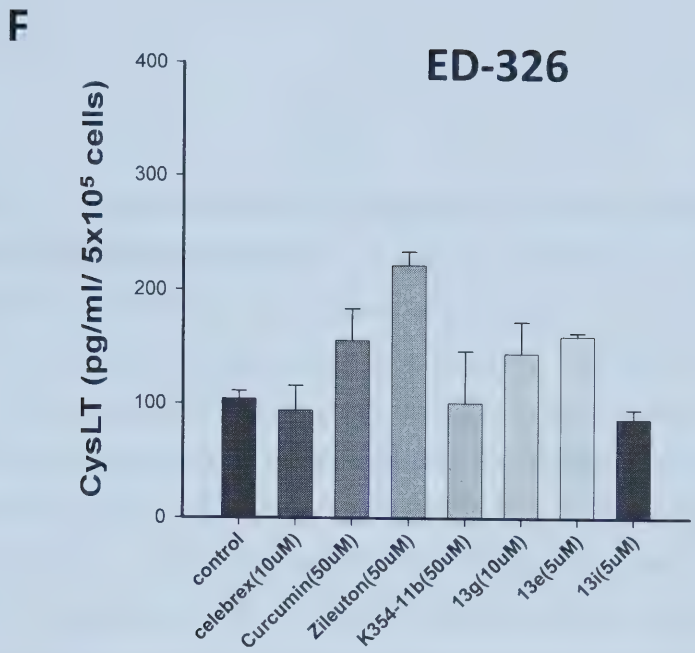

ED-273b
TXB_2 (pg /ml/ 5x10^5 cells)
700
600
500
400
300
200
control
2NTX-99(50uM)
Curcumin(50uM)
K354-11b(50uM)
K354-11b(25uM)
13g(5uM)
13g(10uM)
13e(1uM)
13e(5uM)
13i(1uM)
13i(5uM)

Figure 2-2.


F
ED-326
CysLT (pg/ml/ 5x10^5 cells)
400
300
200
100
0
control
celebrex(10uM)
Curcumin(50uM)
Zileuton(50uM)
K354-11b(50uM)
13g(10uM)
13e(5uM)
13i(5uM)

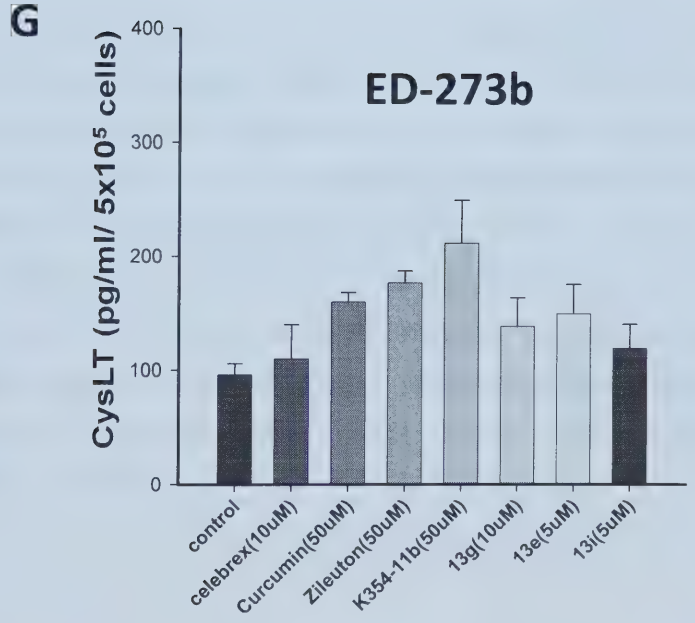

G
ED-273b
CysLT (pg/ml/ 5x10^5 cells)
400
300
200
100
0
control
celebrex(10uM)
Curcumin(50uM)
Zileuton(50uM)
K354-11b(50uM)
13g(10uM)
13e(5uM)
13i(5uM)

Figure 2-3. Cytotoxic effects of compounds on human glioblastoma cell lines and normal human astrocytes. **A,** ED-326, ED-273b, and normal human astrocyte cell lines seeded in 96-well plates at a density of 5 $x10^3$ cells/well were treated with increasing concentrations of k-354-11b, 13g, 13e, 13i, celecoxib (celebrex), curcumin, and NDGA (5-LOX inhibitor) in the presence of 0.5% FBS for 24 h. Dose-response curves were established by measuring the absorbance of the formazan dye using a XTT cytotoxic assay kit. Results shown were plotted as the percentage (%) cytotoxicity mean ± SEM of three triplicate wells of three independent experiments. **B,** A panel of human short-term and long-term-established glioblastoma cell lines and normal human astrocytes (NHA) seeded in 96-well plates at a density of 5 x 10^3 cells/well were treated with increased concentrations of 13e and 13i in the presence of 0.5% FBS for 24 h. Cell-based screening of the cytotoxicity curve of IC_{50} was established using a XTT cytotoxic assay kit. Columns shown were plotted as the average ± SEM of three triplicate wells of two independent experiments. **C,** ED-326 (left column) and ED-273b (right column) cells (2 x 10^4 cells/well) were pretreated with increasing concentrations of chemodrugs: temozolomide (TMZ), etoposide (VP16) or cisplatin (CDDP) in the presence of 2% FBS for 24 h followed by addition of 2.5 μM or 5 μM 13e for another 48 h to determine the additive effect of their combination using a XTT cytotoxic assay kit. Results shown were plotted as the percentage (%) cytotoxicity mean ± SEM of three triplicates wells of three independent experiments.

Figure 2-3.

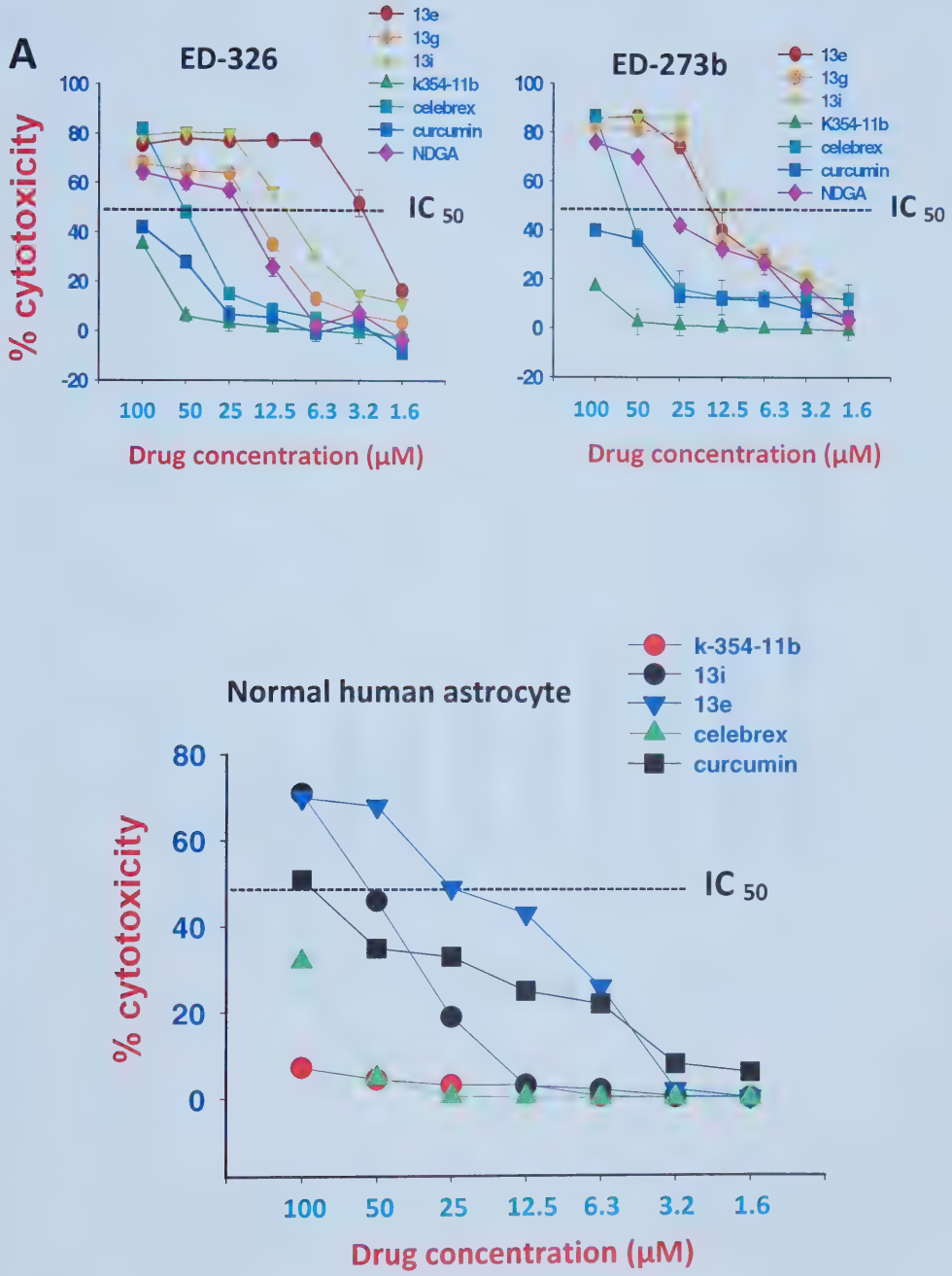

A
ED-326
ED-273b
% cytotoxicity
IC 50
Drug concentration (μM)
100 50 25 12.5 6.3 3.2 1.6
13e
13g
13i
k354-11b
K354-11b
celebrex
curcumin
NDGA
Normal human astrocyte
k-354-11b
13i
13e
celebrex
curcumin

Figure 2-3.

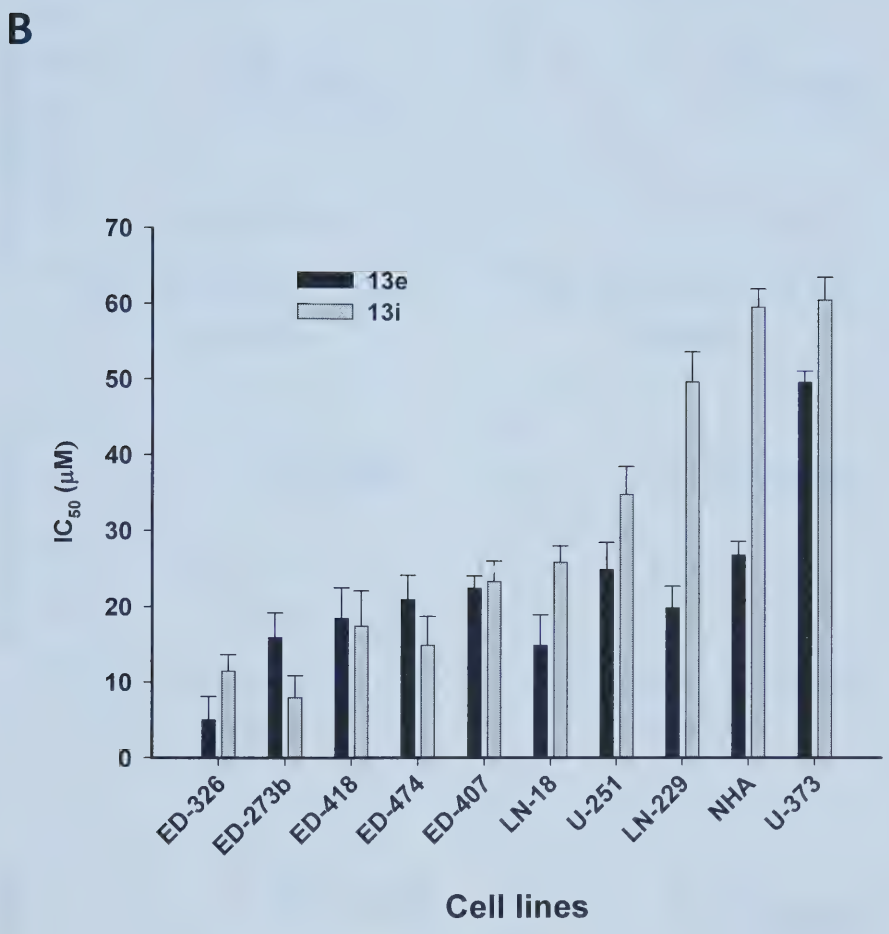

B
13e
13i
IC50 (μM)
70
60
50
40
30
20
10
0
ED-326
ED-273b
ED-418
ED-474
ED-407
LN-18
U-251
LN-229
NHA
U-373
Cell lines

Figure 2-3.

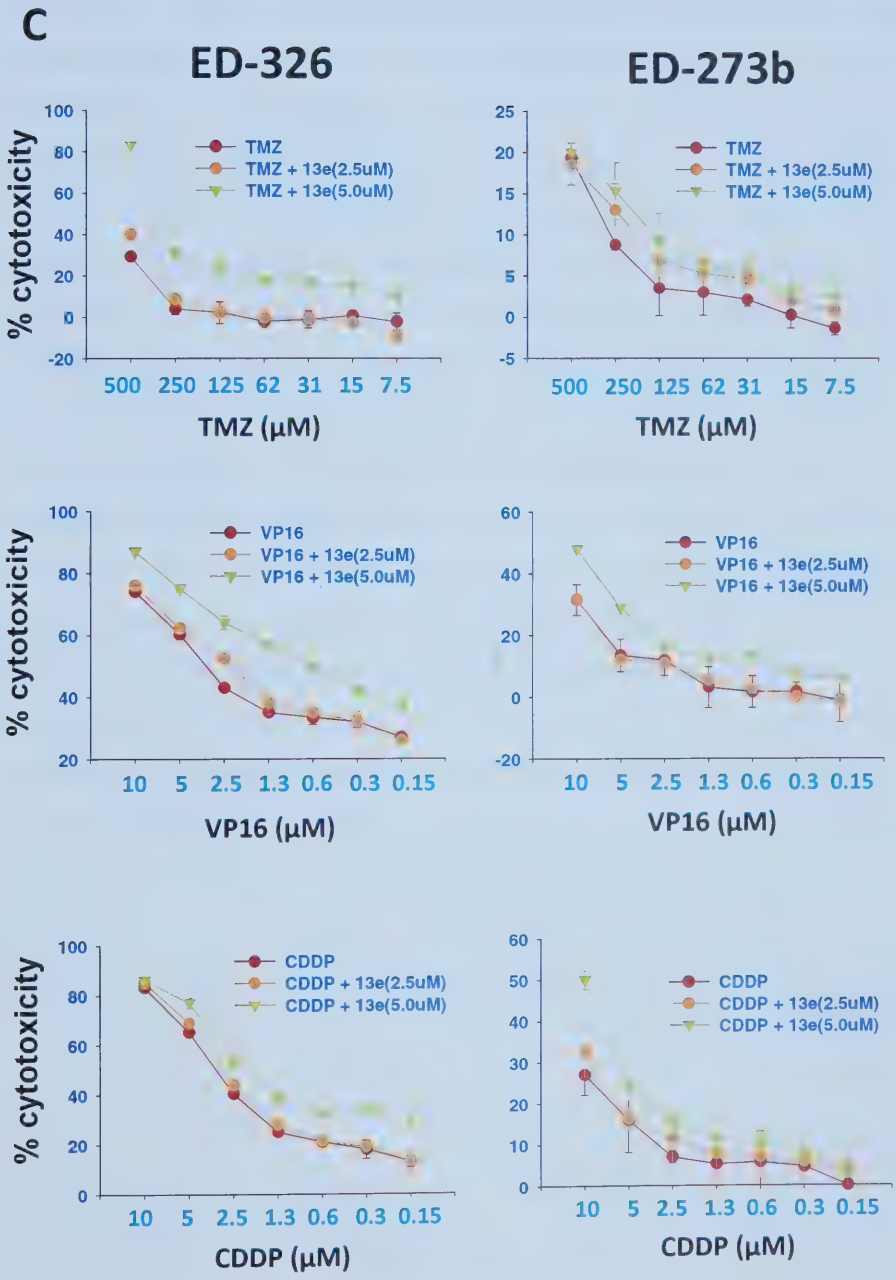

C
ED-326
ED-273b
% cytotoxicity
TMZ
TMZ + 13e(2.5uM)
TMZ + 13e(5.0uM)
500 250 125 62 31 15 7.5
TMZ (μM)
VP16
VP16 + 13e(2.5uM)
VP16 + 13e(5.0uM)
10 5 2.5 1.3 0.6 0.3 0.15
VP16 (μM)
CDDP
CDDP + 13e(2.5uM)
CDDP + 13e(5.0uM)
CDDP (μM)

Figure 2-4. Analysis of apoptosis and cell cycle phase distribution induced by compounds in ED-273b and ED-326 glioblastoma cells. **A,** ED-273b and ED-326 cells, seeded at a density of 5 x 10^6 per dish, were treated with 13e, 13i or 13g at selected concentrations (2.5 to 10 μM) or vehicle control (0 μM) for 48 h. The percentage of apoptotic cell death induced by the drug treatment was determined using Annexin-V and propidium iodide staining followed by flow cytometric analysis. Columns shown were plotted as the percentage (%) Annexin-V positive mean ± SEM duplicates tubes of three independent experiments. **B,** ED-273b and ED-326 cells (5 x 10^6 cells/dish) were treated with 40 μM of 13e, 13i or 13g for 48 h. The cells were fixed and permeabilized with 80% ethanol, stained with propidium iodide, and analyzed by flow cytometry. The percentages (%) for G_0/G_1, S, G_2/M and sub-G_1 phase of the cell cycle were calculated based on the corresponding DNA content of the histograms. Results shown are mean values ± SEM of duplicates tubes of three independent experiments. **C,** Western blot analysis of cells treated with compounds 13g and 13e for cyclin D1 expression, a biomarker marker for G_0/G_1 phase. Anti-ERK1/2 antibody was used as a total protein loading control.

Figure 2-4.

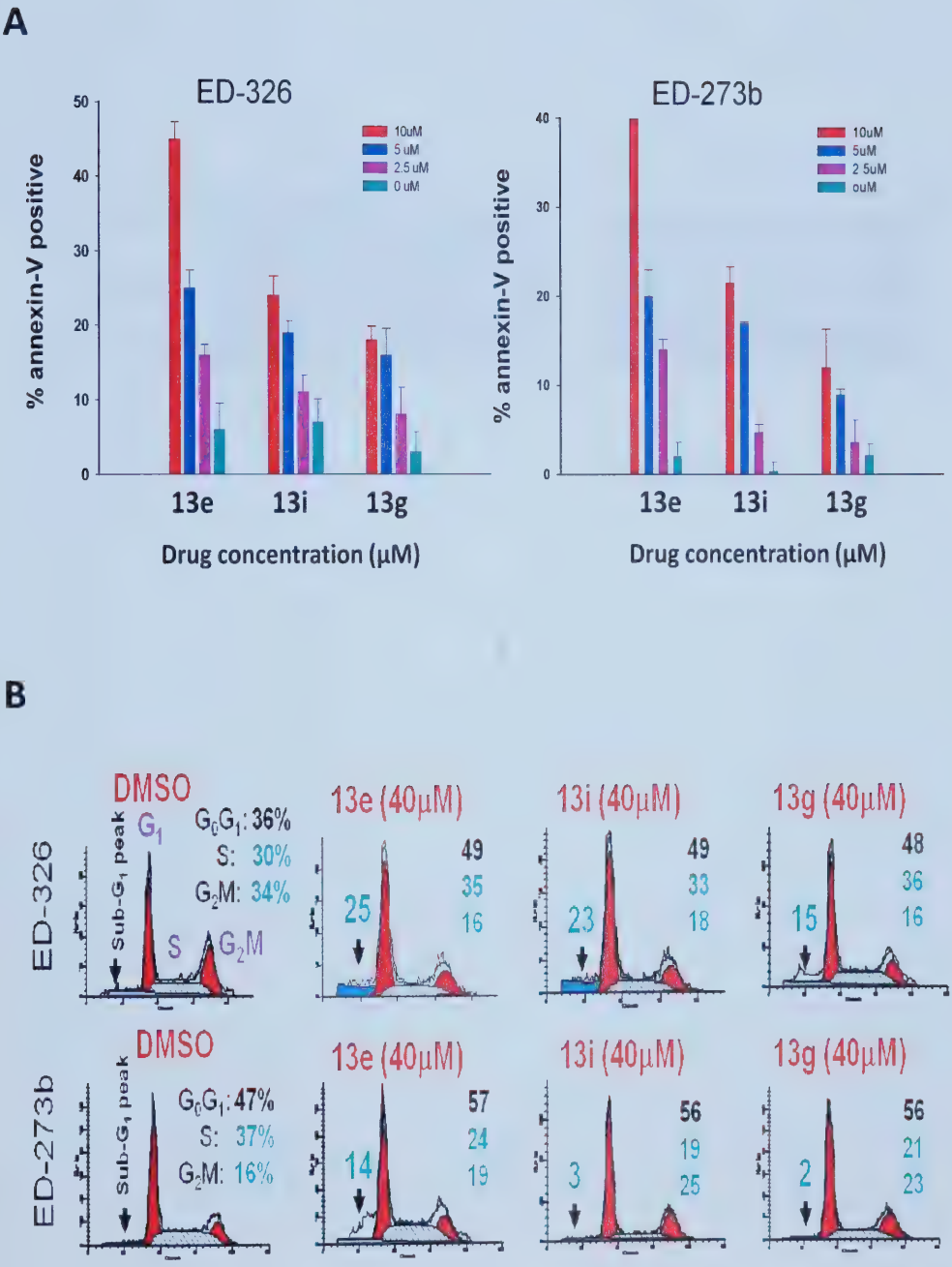

A
ED-326
% annexin-V positive
10uM
5 uM
2.5 uM
0 uM
13e
13i
13g
Drug concentration (μM)
ED-273b
% annexin-V positive
10uM
5uM
2 5uM
ouM
13e
13i
13g
Drug concentration (μM)
B
ED-326
DMSO
Sub-G1 peak
G1
S
G2M
G0G1: 36%
S: 30%
G2M: 34%
13e (40μM)
25
49
35
16
13i (40μM)
23
49
33
18
13g (40μM)
15
48
36
16
ED-273b
DMSO
Sub-G1 peak
G0G1: 47%
S: 37%
G2M: 16%
13e (40μM)
14
57
24
19
13i (40μM)
3
56
19
25
13g (40μM)
2
56
21
23

Figure 2-4.

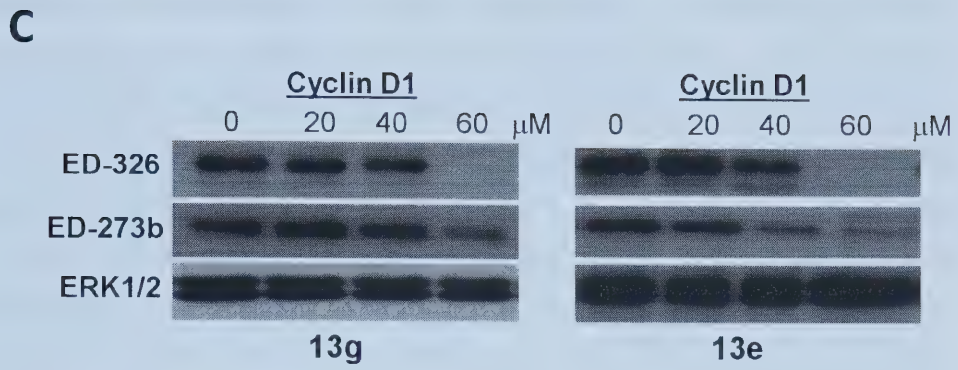
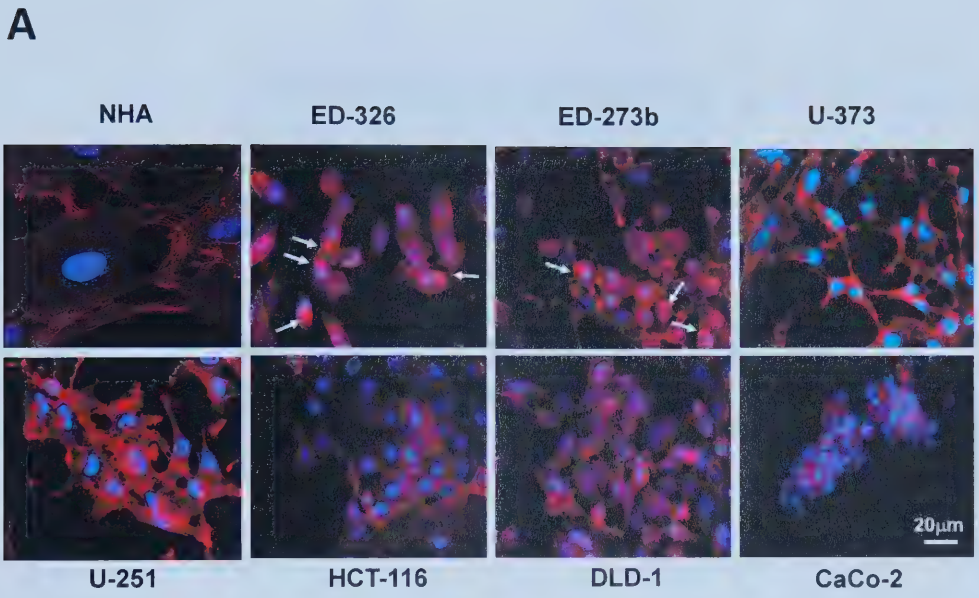

C
Cyclin D1
0 20 40 60 μM
ED-326
ED-273b
ERK1/2
13g
Cyclin D1
0 20 40 60 μM
13e

Figure 2-5. Subcellular β-catenin proteolytic cleavages induced by dual inhibitors in glioblastoma and colon cancer cells. A, Endogenous β-catenin protein expression profile in normal human astrocytes (NHA), glioblastoma ED-326, ED-273b, U-373 and U-251 cells, and human colon HCT-116, DLD-1, and $CaCo_2$ cells. Cells grown on 8-well chamber slide were fixed in 4% paraformaldehyde, permeabilized in 0.2% TX-100, and immunostained with anti-β-catenin followed by CY3 (red)-conjugated secondary antibody and countered stained with DAPI (blue). Arrows depict punctuate staining of β-catenin in the cytoplasm on ED-326 and ED-273b glioblastoma cells. **B,** Phase-contrast photomicrographs of ED-326 and ED-273b glioblastoma cells treated with compounds 13e with increasing concentrations (5-25 μM) for 4 h. The images shown are representative of three separate experiments. **C**, ED-326 (upper panel) and ED-273b (lower panel) cells were stimulated with various doses (0 – 80 μM) of dual inhibitors 13g or 13e for 24 h. The cell lysates were immunoblotted for the inhibitor-induced β-catenin cleavages using anti-β-catenin and anti-E-cadherin antibodies. Anti-α-catenin antibody serves as a loading control. **D**, ED-326 (left panel) and ED-273b (right panel) cells were stimulated with 60 μM of compounds 13g or 13e for the periods of time indicated. The cell lysates were immunoblotted for the inhibitor-induced β-catenin cleavages using anti-β-catenin antibody. **E**, Prostate PC-3 and colon HCT-116 cancer cells were untreated (control) or treated with 100 μM celecoxib (CXB), 50 μM curcumin (Cur), compounds 13g, 13e, 13i at selected concentrations of 80 μM, 60 μM, 60 μM, respectively, for 24 h. The cell lysates were immunoblotted with anti-β-catenin antibody for the induction of β-catenin cleavages, and anti-ERK1/2 antibody for total protein loading control.

Figure 2-5.


A
NHA
ED-326
ED-273b
U-373
20μm
U-251
HCT-116
DLD-1
CaCo-2

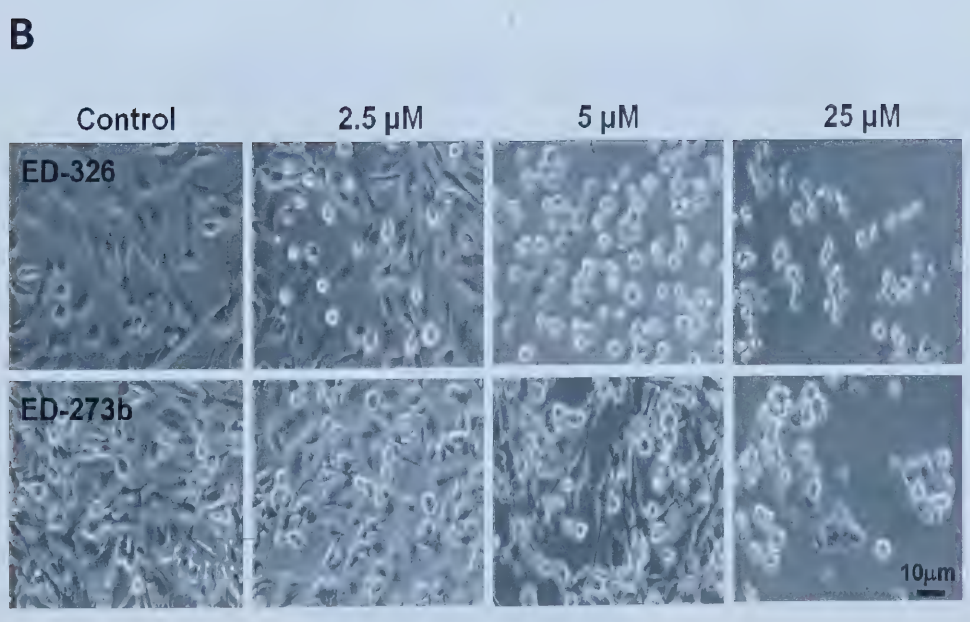

B
Control
2.5 μM
5 μM
25 μM
ED-326
ED-273b
10μm

Figure 2-5

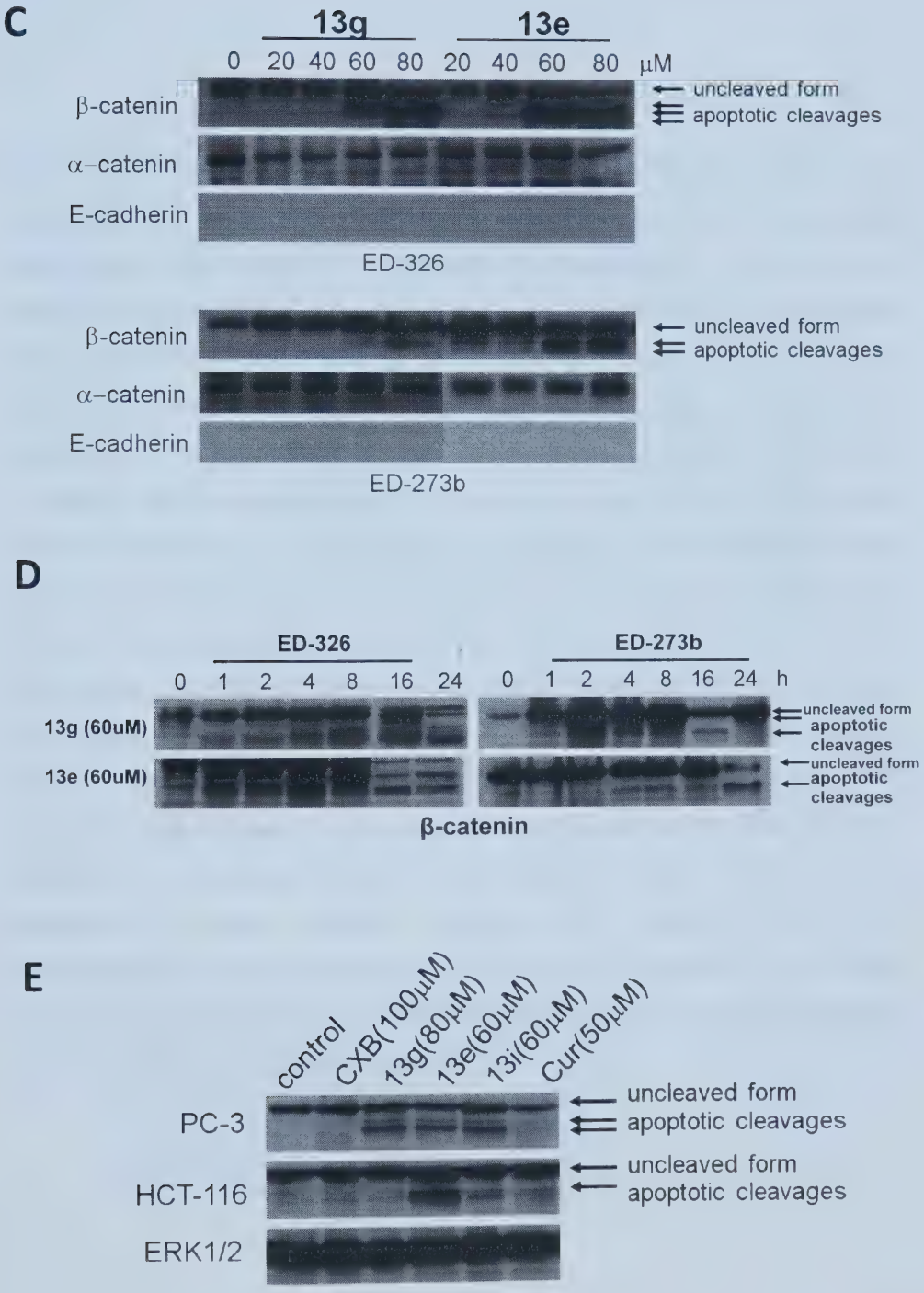
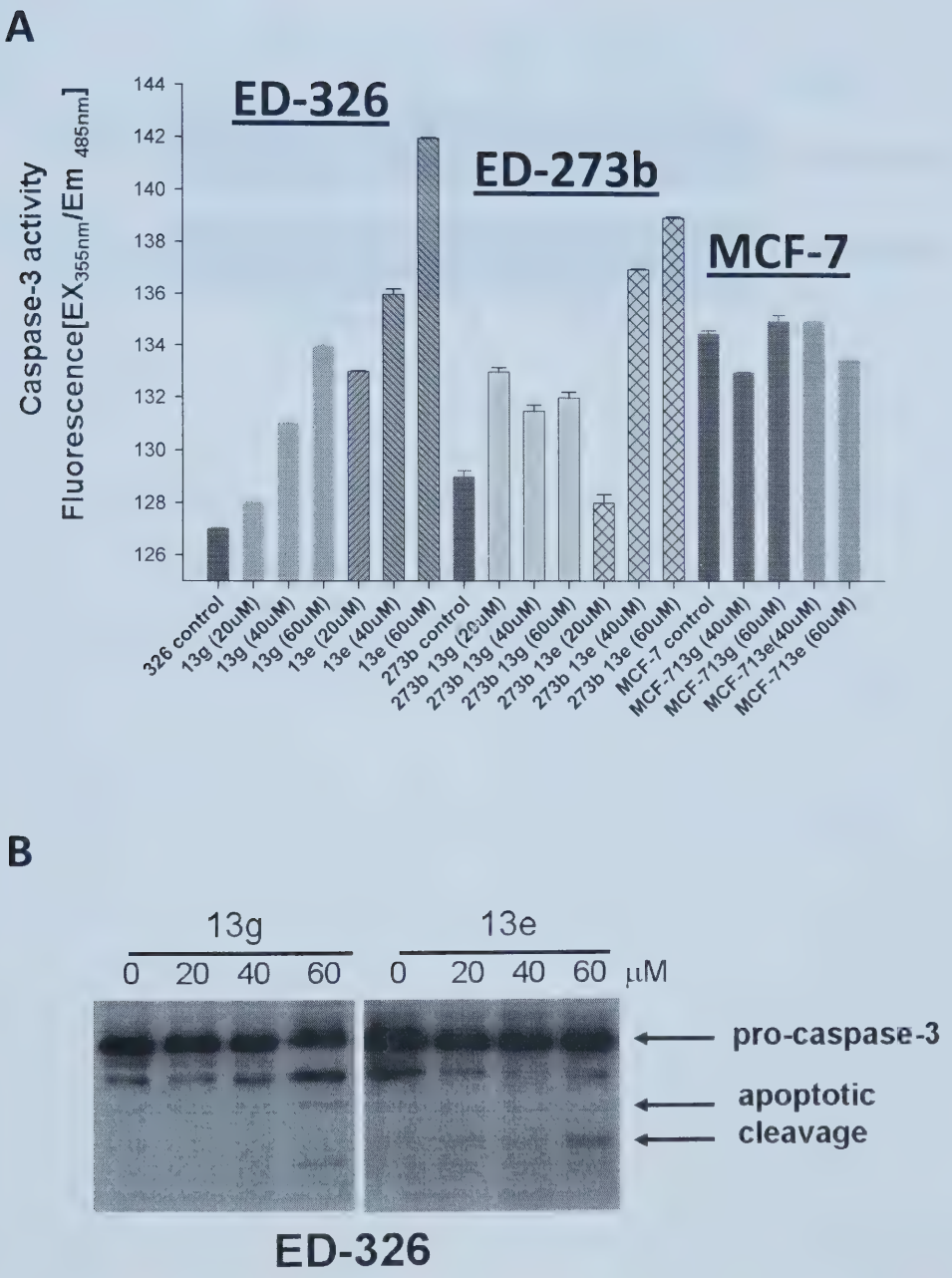
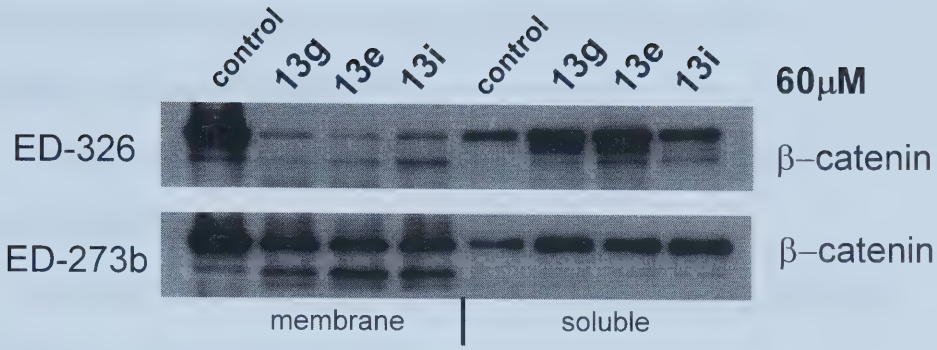

C
13g
13e
0 20 40 60 80 20 40 60 80 μM
β-catenin
α–catenin
E-cadherin
uncleaved form
apoptotic cleavages
ED-326
β-catenin
α–catenin
E-cadherin
uncleaved form
apoptotic cleavages
ED-273b
D
ED-326
ED-273b
0 1 2 4 8 16 24 0 1 2 4 8 16 24 h
13g (60uM)
13e (60uM)
uncleaved form
apoptotic cleavages
uncleaved form
apoptotic cleavages
β-catenin
E
control
CXB(100μM)
13g(80μM)
13e(60μM)
13i(60μM)
Cur(50μM)
PC-3
HCT-116
ERK1/2
uncleaved form
apoptotic cleavages
uncleaved form
apoptotic cleavages

Figure 2-6. Caspase-3-mediated proteolysis of β-catenin induced by compounds 13g and 13e in glioblastoma cells. ED-326 and ED-273b primary glioblastoma cells and MCF-7 breast cancer cells (caspase-3 null cells) were treated with compounds 13g and 13e at increasing concentrations (20, 40 and 60 μM) or untreated control (0μM) for 24 h. The cells were scraped and lysed; the supernatants were stored at -20°C until used. **A,** The caspase-3 activity was quantified in the supernatant at equal concentrations of the cell lysates (200 μL/sample) using a caspase-3 activity fluorimetric assay kit (Roche Diagnostics, Laval, QC) according to the manufacturer's instructions. The final products were measured on a fluorescence microplate reader (Thermo Labsystems Fluoroskan Ascent FL) with excitation filter 355 nm and emission filter 485 nm and the fluorescence value was plotted versus compounds concentrations. **B,** An aliquot of the same ED-326 cells was analyzed by western blot using anti-caspase-3 antibody. **C,** ED-326 and ED-273b glioblastoma cells were treated with compounds 13g, 13e and 13i at a concentration of 60 μM for 24 h. The cells were subjected to biochemical analysis using detergent Triton X-100 for phase separation to obtain cytosolic (Triton X-100 soluble fraction) and membrane/nuclear fractions (Triton X-100 insoluble fraction). The isolated fractions of cell lysates were immunoblotted for the inhibitor-induced β-catenin cleavages using an anti-β-catenin antibody.

Figure 2-6


A
Caspase-3 activity
Fluorescence[EX355nm/Em 485nm]
144
142
140
138
136
134
132
130
128
126
ED-326
ED-273b
MCF-7
326 control
13g (20uM)
13g (40uM)
13g (60uM)
13e (20uM)
13e (40uM)
13e (60uM)
273b control
273b 13g (20uM)
273b 13g (40uM)
273b 13g (60uM)
273b 13e (20uM)
273b 13e (40uM)
273b 13e (60uM)
MCF-7 control
MCF-713g (40uM)
MCF-713g (60uM)
MCF-713e(40uM)
MCF-713e (60uM)
B
13g
13e
0 20 40 60
0 20 40 60 μM
pro-caspase-3
apoptotic
cleavage
ED-326

C


control
13g
13e
13i
control
13g
13e
13i
60μM
ED-326
β–catenin
ED-273b
β–catenin
membrane
soluble

2.5. References

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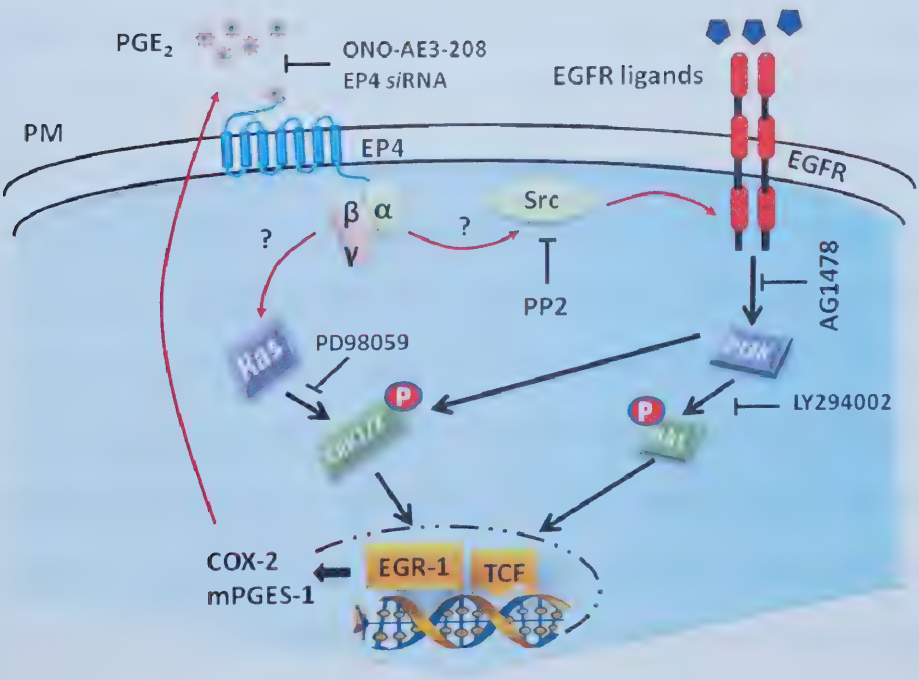
Chapter 3

PGE_2-EP4 Signaling Enhances Glioblastoma Cell Proliferation and Invasion through Transactivation of EGFR and ERK/Akt Activation



3.1. Introduction

Prostaglandin E_2 (PGE_2) is a short-lived lipid autacoid that is involved in many physiological and pathological processes such as inflammation, pain, fever, and carcinogenesis (1-3). It is synthesized from arachidonic acid (4) by the action of cyclooxygenases and is the predominant metabolite of cyclooxygenase-2 (COX-2). Ample evidence suggests that COX-2 plays a pivotal role in promoting cell growth and invasion in various types of cancers including brain (5), colon (6), prostate (7) and lung (8). Elevated COX-2 expression observed in these malignancies has been found to correlate with a poorer patient survival (9-11). Furthermore, COX-2-derived PGE_2 promotes tumor cell growth in colon cancer (6), cholangiocarcinoma (12) and medulloblastoma (5) via multiple downstream signaling pathways. Treatment with selective COX-2 inhibitors suppressed tumor cell growth *in vitro* (13, 14) including glioblastoma (15, 16) and animal models of colorectal cancer (17, 18). In this regard, selective inhibition of COX-2 has emerged as a prominent therapeutic target for chemopreventive or cancer therapy.

PGE_2 exerts its diverse biological activities by binding to a subfamily of G protein-coupled receptors (GPCRs), named EP1, EP2, EP3 and EP4. The biological function of these receptor subtypes varies depending on their expression profile and cell specificity. Recently, PGE_2 was shown to enhance cell proliferation and invasion via epidermal growth factor receptor (EGFR) signaling through EP receptor cross-talk, by activating other signaling molecules, including c-Src, β-catenin and matrix metalloproteinases (MMPs) in head and neck

squamous cell carcinomas (19), colon cancers (4, 20) and cholangiocarcinomas (12). To date, there is limited information on PGE_2 signaling or EP receptor biological function in glioblastoma cells. Defining the underlying mechanism by which PGE_2 mediates cell growth in GBM may provide a better understanding of glioma biology and alternative therapeutic strategies against gliomas.

Glioblastoma multiforme (GBM), with a median survival of 12 to 15 months (21) is the most devastating tumor of the central nervous system (CNS) and remains refractory to current standard care consisting of surgery, radiotherapy and concomitant/adjuvant temozolomide. Glioblastoma is characterized histologically by nuclear pleomorphism, pseudopalisading necrosis, and microvascular proliferation and genetically by various multiple mutations and amplifications such as mutation of *PTEN* (phosphatase and tensin homologue deleted in chromosome 10) and *EGFR* amplification or overexpression (22). These genetic alternations ultimately lead to aberrant activation of signaling pathways such as Ras/mitogen-activated protein kinase (ERK1/2) and Akt/phosphatidylinositol-3 kinase (PI3K), resulting in the promotion of cell proliferation, survival, and invasion (23, 24). Interestingly, *Akt* and *Ras* are not mutated in human GBM (25) but their activities are frequently increased (26, 27) and combined activation of Akt and Ras induces GBM formation in mice (28), suggesting that increased Akt and ERK1/2 activities are due to upstream factors. Furthermore, microarray and immunohistochemical analysis studies have showed that EP4 receptor is overexpressed in the perinecrotic regions of high grade but not lower grade glioma tumors and that EP4 expression correlates with patient

survival in GBM (29). These studies indicate both the Ras/Akt cascades and the EP4 receptor may be critical effectors of GBM development. Moreover, increased biosynthesis of PGs, most notably PGE_2, has been detected in human intracranial tumors and brain metastasis (30-32). Recently, PGE_2 has been shown to promote tumor growth via the EP1 and EP3 receptors but not the EP4 in medulloblastoma, a common childhood embryonic tumor of the cerebellum (5). Whether PGE_2 affects GBM cell growth and invasion has not been fully established.

To delineate the molecular mechanisms by which PGE_2 induces GBM cell proliferation and progression, we used early passage primary cultures of GBM derived from the biopsy specimens as a model for this study. Here, we report for the first time to our knowledge that COX-2-derived PGE_2 transactivates EGFR signaling and triggers downstream ERK1/2 and Akt signaling pathways, thereby resulting in cell proliferation and invasion. This PGE_2-induced activation of EGFR requires the EP4 receptor and includes the involvement of an intracellular Src kinase mechanism.

3.2. Materials and Methods

Cell Culture and Reagents

Primary cultures of ED-189, ED-273b, ED-326, ED-432b, ED-486, ED-487, ED-488, ED-490, ED-493, ED-499 human glioblastoma cell lines derived from patients were established and characterized as previously described (33). Normal human fetal astrocyte (NHA) cell line was a generous gift from Dr.

V.Wee Yong (University of Calgary, Calgary, Canada). The long-term established glioma cell lines LN-71, LN-18, LN-229, U-251, U-87 and U-373 were generous gifts from Dr. Charles Hao (Emory University School of Medicine, Atlanta, GA). All glioblastoma cell lines were maintained in Dulbecco's modified Eagle's medium and F-12 nutrition mixture (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 units/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Norma human fetal astrocytes were maintained in RPMI 1640 media in 75 cm^2 culture flasks precoated with 1mg/mL of poly-L-lysine (Sigma-Aldrich) and used within the first 3-4 passages. Butaprost, sulprostone, 16,16-dimethyl PGE_2, PGE_1-OH, AH6809 and LY 294002 were purchased from Cayman Chemical (Ann Arbor, MI). The EGFR tyrosine kinase inhibitor AG1478, Src kinase inhibitor PP2, extracellular signal-regulated kinase kinase 1 (MEK1) PD98059, PI3K inhibitor PI-103 and mTOR inhibitor rapamycin were obtained from Calbiochem (La Jolla, CA). ONO-AE3-240 (EP3 antagonist) and ONO-AE3-208 (EP4 antagonist) were a general gift from ONO Pharmaceutical Co. Ltd. (Chuoku, Osaka, Japan). Their structures and pharmacokinetic properties have been well-characterized in several types of tumor *in vitro and in vivo* (34, 35). The selective COX-2 inhibitor, celecoxib (CXB) was purchased from LKT laboratories, Inc. (St. Paul, MN). Epidermal growth factor was purchased from StemCell Technologies Inc. (Vancouver, BC). Unless otherwise indicated, all other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada). For all inhibitors, dimethyl sulfoxide (DMSO) was used as a solvent.

Western Blot and Immunoprecipitation

Cells were serum-starved for 24 h prior to the immunblotting experiments. In some cases, cells were pretreated with various eicosanoid agonists, antagonists or kinase inhibitors as indicated in the figure legends. After experimental treatment, the cells were washed with PBS, scraped in PhosphoSafe Extraction reagent (Novagen, Gibbstown, NJ) containing 1mM PMSF and protease inhibitor tablets (Roche Diagnostics, Laval, Quebec, Canada) lysis buffer for 30 min on ice and centrifuged at 14,000 rpm for 15 min at 4°C. The protein concentration of the cell lysate was determined using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Fifty micrograms (50μg) of protein were resolved on SDS-PAGE, transferred to a 0.45 μm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked with 5% non-fat dry milk in TTBS (20 mM Tris-HCL[pH.7.5]+ 150 mM NaCl + 0.5% Tween-20) buffer for 1 h and then incubated overnight at 4°C with the following primary antibodies: anti-EGFR (mouse mAb, IP specific), anti-EGFR (rabbit polyclonal antibody), anti-pEGFR^{Tyr845}, anti-Src, anti-pSrc^{Tyr416}, anti-phospho-tyrosine (PY-100), anti-phospho-p44/42 MAPK (pERK1/2), anti-Akt, anti-pAkt^{Ser473}, anti-TCF-4 and anti-EGR-1 from Cell Signaling Technology (Danvers, MA); anti ERK1/2 from Stressgen Bioreagents (Ann Arbor MI); anti-COX-2, anti-mPGES-1 and anti-EP4 from Cayman Chemical (Ann Arbor, MI); and anti-cyclin D1 from Calbiochem (La Jolla, CA). After three washes with TTBS (10 min each wash), the membrane was incubated with appropriate horseradish peroxidase-conjugated secondary antibodies for 45 min at room temperature and followed by three washes with

TTBS. The immunoreactive protein bands were visualized by Western Lighting® chemiluminescence reagent plus (PerkinElmer, Boston, MA). To ensure equal loading, the same blot was stripped with Restore® western blot striping buffer (Thermo-scientific, Rockford, IL) for 45 min. washed in PBS and reprobed with non-phosphorylated appropriate antibodies for loading control. For immunoprecipitation, fresh cell lysates were incubated overnight with anti-EGFR or anti-c-Src antibodies at 4°C. After the incubation, 50 μL of protein G Sepharose slurry (GE Bio-Science, Inc. Baie d'Urfe, QC) was added and incubated for another 1 h at 4°C. The pellets were washed in cold lysis buffer 3 times and resuspended in 2x Laemmli sample buffer for western blot analysis.

Reverse Transcription-PCR (RT-PCR)

Total cellular RNA was extracted from 5 x 10^6 cells using Trizol® reagent (Invitrogen, Carlsbad, CA) and purified according to the method supplied by the manufacturer. In brief, single-stranded cDNA was synthesized in a reaction containing 1 μg of total RNA and Superscript III transcriptase (Invitrogen, Carlsbad, CA) for 30 min. at 48°C and amplified with Platinum Tag DNA polymerase (Invitrogen, Carlsbad, CA) in cycle conditions as follows: 35 cycles consisting of an initial denaturation of 94°C for 2 min, then 94°C for 15 sec., 54°C for 30 sec. and 70°C for 1 min. The following primer sets for each EP receptor subtype (amplicon sizes are in parentheses) were used for amplification: EP_1 forward [5'-GGTATCATGGTGGTGTCGTG-3'] and reverse [5'- GGCCTC TGGTTGTGCTTAGA-3'(324bp)]; EP_2 forward [5'- GCCACGATGCTCATGCT

CTTCGC-3'] and reverse [5'- CTTGTGTTCTTAATGAAATCCGAC-3'(655bp)]; EP_3 forward [5'- CGTGTCGCGCAGCTACCGGCG-3'] and reverse [5'- CGG GCCACTGGACGGTGTACT-3' (398bp)]; EP_4 forward [5'- GGGCTGGCTGTC ACCGACCTG-3'] and reverse [5'- GGTGCGGCGCATGAACTGGCG-3' (485 bp)]. A portion of each reaction was analyzed on a 1.2% agarose gel.

Trypan Blue Exclusion

Subconfluent cells were serum-starved for 24 h, pretreated with various inhibitors for 1 h and followed by PGE_2 (2.5 μM) treatment for 4 days. After treatment, cells were harvested with trypsin, stained with trypan blue, and counted manually with a hemacytometer. Cells that excluded trypan blue were considered viable.

Proliferation and XTT Assays

The biological effect of PGE_2 with respect to cell proliferation was determined by measuring the incorporation of [^{3}H] thymidine into cellular DNA. ED-273b and U-251 cells (1x 10^5cells/well) were seeded in 24-well plates and incubated overnight to attain 75-80% confluence. Cells were serum-starved for 24 h and then treated with PGE_2, EP2 and EP4 agonist and antagonist or a combination of both in the fresh medium containing 0.5% fetal calf serum for 3 d which was renewed daily. 2 μCi of methyl-[^{3}H] thymidine (GE Biosciences, Baie d'Urfe, Quebec) was added to each well and incubated for 4 h prior to termination of each experiment. The cells were rinsed 3 times with cold PBS, lysed with 500

μL of 0.5 N NaOH, immediately neutralized with 500 μL of 0.5 N HCL and the radioactivity was measured on β scintillation counter (Beckman Coulter Inc, Mississauga, ON). To measure the cytotoxic effect of celecoxib, a cell proliferating kit II (XTT) (Roche Molecular Biochemicals, Laval, QC) assay was used to determine cell viability after treatment with CXB. ED-273b and U-251 cells ($5x10^3$cells/well) were seeded in 96-well plates overnight. After being serum-starved for 24 h, these cells were stimulated with increasing concentrations of CXB (1.56-100μM). After 24 h, 50 uL of XTT labeling mixture (5mL XTT labeling reagent with 0.1mL electron labeling reagent) was added to each well and incubated for 4 h at 37°C. The percentage of cytotoxic cells was calculated by standardization to untreated controls.

Measurement of PGE_2 Levels

Early passage primary cultures of 10 glioblastoma cell lines; 6 long–term established glioma cell lines and normal human fetal astrocyte (36) cell line were seeded in 12-well plates ($5x10^5$cells/well) for overnight. After 24 h incubation, the culture supernatants were harvested and centrifuged at 2,500 rpm for 5 min. and thereafter subjected to PGE_2 levels determination using a PGE_2 EIA kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer's instructions. The absorbance of the yellow dye was directly quantified with the use of a Benchmark microplate reader (Bio-Rad Laboratory, Mississauga, ON) set at 409 nm against a reference wavelength of 650 nm. PGE_2 concentration was calculated using the measured optical density of the samples against a standard curve.

SiRNA Knockdown Assay

ED-273b cells were transiently transfected with an EP4 *SMART*pool siRNA (a mixture of four different siRNA targeted against EP4) or a control siRNA (Dharmacon Inc. Chicago, IL) using Lipofectamine reagent (Invitrogen, Carlsbad, CA). Seventy-two hours after the transfection, the cells were treated with 2.5 μM PGE_2 or 5 μM butaprost for 15 min. Gene knockdown of EP4 and phosphorylation of ERK1/2 were analyzed by western blot. Cell proliferation of the ED-273b transfected cells was also measured by [^{3}H] thymidine incorporation. Results were plotted as the means ± SD ($^*p<0.05$ vs. control) of the two independent experiments.

Invasion Assay

The ED-273b tumor cell *in vitro* invasion study was assessed using BD BioCoat™Growth Factor Reduced Matrigel®Invasion Chambers (BD Biosciences, Bedford, MA). Briefly, $2.5x10^4$ cells in 0.5 mL serum-free media were placed in the upper chamber of 8.0 μm pore of the PET membrane. The lower chamber was filled with serum-free media in the presence of 2.5 μM PGE_2 with or without the specific inhibitors. After 24 h incubation, cells from the upper surface of the filter insert were removed with a cotton swab and cells adhering to the under surface of the filter were fixed and stained using the HESM staining kit (Fisher Scientific, Ottawa, ON) according to the manufacturer's protocol. The cells were counted under the microscope and the number of cells that invaded the Matrigel®

Chambers was determined and expressed as the mean ± SD of assays performed in triplicate.

Statistical Analysis

The data were expressed as the mean ± SD. Student's *t*-test was used to compare data between the 2 groups. One-way ANOVA followed by Bonferroni's test post hoc was applied to compare data between ≥ 3 and an unpaired two-group *t*-test for single comparisons using a SPSS 14 statistical software. The level of statistical significance was set at $P < 0.05$.

3.3. Results

COX-2 Expression and PGE_2 Production Are Significantly Increased in Primary Glioblastoma Cells.

While characterizing the pharmacological properties of dual COX-2/5-LOX inhibitors (in Chapter 2) in primary cultures of glioblastoma cells, we noted an increase in PGE_2 levels in numerous of the primary glioblastoma cell lines, reflecting growth correlation. This observation prompted us to investigate whether PGE_2 plays a role in cell proliferation and growth. Previous studies have shown that human intracranial tumors secrete large amounts of PGE_2 (30, 32). To assess the relevance of this finding, we screened a panel of available early passage primary cultures of human glioma established previously in our laboratory (33) and other long-term established glioma cell lines. The results of this study

demonstrate that primary cultures of GBM cells such as ED-273b, ED-326 and ED-490 secreted significant amounts of PGE_2, ranging from 7,000 to 32,000 pg/mL/5x 10^5 cells. Lower levels of PGE_2 (2,000 to 5,000 pg/mL) were seen in ED-487, U-87 and U-373 cells, while little or no PGE_2 was produced in other GBM cell lines (e.g. U-251) and including NHA (**Figure 3-1A**). Next, we investigated whether the increased basal PGE_2 production correlates with constitutive COX-2 expression. Western blot analysis demonstrates that there was a strong correlation of COX-2 expression with PGE_2 production in ED-273b cells followed by ED-326, ED-489, U-87 and LN-229; whereas COX-2 expression was not detected in U-251 cells (**Figure 3-1B**). These results support the premise that COX-2-derived PGE_2 may play a critical role in modulation of glioma cell proliferation and progression.

PGE_2 Induces Glioblastoma Cell Proliferation Irrespective of COX-2 Expression

To investigate the underlying molecular mechanism by which PGE_2 promotes cell proliferation, we first evaluated the cytotoxic effect of the selective COX-2 inhibitor celecoxib (CXB) in ED-273b and U-251 cells and examined the effect of PGE_2 on cell proliferation. Interestingly, celecoxib treatment induced cytotoxicity in both ED-273b (COX-2 expressing) and U-251 (barely detectable COX-2 levels) cells in a dose-dependent fashion, with minimal cytotoxic effect (i.e. < 10%) obtained at ~10 μM concentration (**Figure 3-2A**). It has been shown that the physiological dose of CXB is at 10 μM, which had no effect on the cell growth but resulted in 80 – 90% inhibition of PGE_2 production (37). Likewise,

our early dose-response curve study showed that CXB (at 5-10 μM) treatment inhibited 90-95% PGE_2 production in ED-273b cells (data not shown). Our results indicate that the growth of ED-273b and U-251 cells are equally sensitive to the COX-2 inhibitor and that the physiological dose of CXB can be used to eliminate the basal level of the endogenous PGE_2 in ED-273b cells.

Next, we investigated whether addition of exogenous PGE_2 is able to rescue GBM cells from the COX-2-inhibitory effects of celecoxib. For this purpose, we employed 16,16-dimethyl PGE_2, a synthetic long acting and stable analog of PGE_2 (38), which acts as an agonist for most of EP subtype receptors such as EP2, EP3 and EP4. To eliminate the endogenous basal levels of PGE_2, we chose to pretreat cells with 10 μM CXB (a relevant physiological dose which is not toxic to cells) for 24 h in the presence of 0.5% FBS. As shown in **Figure 3-2B**, addition of exogenous PGE_2 rescued (promoted) the growth of ED-273b cells (COX-2 positive) pretreated with CXB when compared to the control levels and near the levels of PGE_2-treated cells without CXB treatment. In contrast, exogenous PGE_2 stimulation failed to promote cell proliferation in the absence of CXB, implicating that the endogenous basal PGE_2 levels was sufficient to support cell proliferation under the steady-state conditions. Interestingly, exogenous PGE_2 enhanced both the growth of U-251 cells (COX-2 negative) in the presence or absence of CXB treatment (**Figure 3-2B**). In addition, PGE_2 exposure (replenished daily) for 4 days increased the number growth of both GBM cell lines by 1.5 fold, compared with untreated controls and significantly rescued the growth of both cell lines pretreated with low dose of 10 μM CXB (**Figure 3-2C**). Furthermore, under

serum-free conditions, PGE_2 exposure caused a dose-dependent increase level of COX-2 expression in ED-273b cells but not in U-251 cells (**Figure 3-2D**). These results indicate that PGE_2 can stimulate GBM cell proliferation and growth via dependency of COX-2 or non-COX-2 expression in glioblastoma cells.

PGE_2 Transactivates of EGFR and Triggers the ERK1/2 and Akt Signaling Pathways.

Ample evidence suggests that PGE_2 promotes cell growth and survival mediated through a GPCR-EGFR cross-talk mechanism, thereafter activating the MAPK and PI-3K kinases signaling pathways (4, 39). To gain further insight into a possible alternative pathway used by ED-273b cells, we examined the ability of PGE_2 to transactivate EGFR signaling cascade (19). To rule out the possible activation of EGFR by PGE_2 via an autocrine loop, we performed a time-course screen (0, 3, 8, 24 h) of PGE_2 production in ED-273b cells under serum free conditions. We found that COX-2-derived PGE_2 was detected in relatively low concentrations in the culture media at 24 h (data not shown). After 24 h of serum starvation, we stimulated ED-273b cells with PGE_2 (2.5 μM) for various time periods and determined EGFR phosphorylation levels by immunoprecipitation of the EGFR followed by western blot analysis with phosphotyrosine-specific antibodies. PGE_2 stimulation caused a time-dependent increase of tyrosine phosphorylation of EGFR (**Figure 3-3A**). Inactivation of EGFR with a specific kinase inhibitor tyrphostin AG 1478 (10 μM, 60 min.) greatly abolished the PGE_2-induced activation of EGFR. Next, we examined whether transactivation of

EGFR by PGE_2 triggers extracellular signal-regulated kinase 1 and 2 (ERK1/2) and Akt (protein kinase B) activities. As shown in **Figure 3-3B**, PGE_2 stimulation induced the phosphorylation of ERK1/2 and Akt in a dose-and-time dependent fashion. The PGE_2-induced activation of ERK1/2 and Akt was maximal at 2.5 μM and 0.1 μM, respectively, and leveled off. Similarly, PGE_2 increased activation of both ERK and Akt at 1 h; and this activation was sustained up to 24 h. To determine whether the PGE_2-induced ERK1/2 and Akt activations is a general phenomenon of glioblastoma cells, we stimulated three other GBM cell lines (one primary and two long-term established) with PGE_2 and examined the downstream effector signaling. As expected, PGE_2 stimulation triggered phosphorylation of ERK1/2 and Akt in primary ED-189 and long-term established LN-229 and U-251 cells (**Figure 3-3C**). This PGE_2-stimulatory effect was abolished or significantly attenuated in cells pretreated with EGFR kinase inhibitor AG 1478 (**Figure 3-3C**). Likewise, the PGE_2-stimulated cell proliferation was significantly diminished in the presence of the EGFR kinase inhibitor AG 1478 as reflected by decreased [^{3}H]-thymidine incorporation into DNA compared with untreated controls of ED-273b and U-251 cells (**Figure 3-3D**). Taken together, these results indicate that PGE_2 triggers the downstream mitogenic ERK1/2 and Akt signaling to promote glioblastoma cell proliferation and growth via transactivation of the EGFR.

PGE_2 Transactivates EGFR via an Intracellular Kinase Src Mechanism.

Recent studies have indicated that PGE_2 transactivates EGFR via an intracellular nonreceptor tyrosine kinase Src mechanism to promote cell growth and invasion in colon cancer (4) and cholangiocarcinoma (12). Inhibition of Src kinase activity was shown to arrest PGE_2-induced cell proliferation and prevented tumor invasion mediated by EGFR activation (39). To investigate the role of Src as a signaling mediator between GPCR (i.e. EP receptor) and EGFR in the context of PGE_2-induced activation of EGFR, we first examined whether PGE_2 activates Src by measuring the activity of c-Src using an active tyrosine phosphorylated Src (Src^{Tyr415}) antibody (40). As shown in **Figure 3-4A** (**upper panel**), PGE_2 dose- and time-dependently induced Src activation which was leveled off after 1 μM and 1 h duration. In addition, pretreatment of ED-273b cells with pharmacologic antagonist of Src tyrosine kinase (PP2), dose-dependently attenuated the PGE_2-induced ERK1/2 activation (**Figure 3-4A**, **lower panel**). Next, we determined whether PGE_2-induced activation of Src is associated with the EGFR or GPCR signaling. For this purpose, we treated ED-273b cells with 2.5 μM PGE_2 for 5 or 15 min and the cell lysates were subjected to immunoprecipitation with an anti-Src antibody followed by western blot analysis using an anti-pEGFR antibody. Treatment with PGE_2 markedly enhanced the binding of Src to EGFR with time as demonstrated by western blot showing the increased phosphorylation of EGFR (**Figure 3-4B**, **top**). However, pretreatment of ED-273b cells with PP2, followed by immunoprecipitation with anti-EGFR antibody and immunoblotting with anti-tyrosine-phosphorylated (PY 100) antibody, resulted in an attenuation of PGE_2-induced phosphorylation of EGFR (**Figure 3-4B**, **bottom**), suggesting that Src is

involved in PGE_2-induced EGFR activation. In addition, pretreatment of ED-273b cells with specific EGFR inhibitor AG1478 showed no appreciable effect on the PGE_2-induced Src phosphorylation whereas, as expected, the Src inhibitor PP2 completely abolished this effect (**Figure 3-4C**). The results suggest that Src is activated prior to the PGE_2-induced transactivation of the EGFR.

To assess the signaling role of Src in PGE_2-induced ERK1/2 activation, we pretreated ED-273b and U-251 cells with pharmacologic antagonists of MEK (PD98059) and Src kinase (PP2) in the presence of PGE_2 for 4-5 days (replenished daily). Cell proliferation and growth were evaluated by [^{3}H] thymidine incorporation and trypan blue exclusion. PD98059 (41) or PP2 treatment in the presence of PGE_2 significantly attenuated PGE_2-induced cell proliferation (cpm) and cell number (**Figure 3-4D**) in both ED-273b and U-251 cells but caused no significant changes in the levels of cell proliferation and cell number when compared with untreated control cells (**Figure 3-4D**), suggesting that the PGE_2-induced ERK1/2 may be stimulated in part through an EGFR-independent mechanism such as GPCR. As expected, PGE_2 treatment alone significantly enhanced cell proliferation and increased cell number by 1.5 to 2-fold compared with untreated controls (**Figure 3-4D**). These results indicate that PGE_2-induced cell proliferation is mediated through transactivation of the EGFR signaling via the Src kinase activity and thereafter leading to activation of the ERK1/2 pathway.

Glioblastoma Cell Growth is Associated with EP4-mediated ERK1/2 and Akt Activation

It is known that PGE_2 exerts its diverse effects through binding to its cognate receptors called EP1, EP2, EP3, and EP4 receptors (42). The specificity of PGE_2-induced biological action depends on the expressing receptor subtype. To characterize the role of these EP receptors in PGE_2-induced cell division, we first investigated the expression profile of EP receptor subtypes in ED-273b and U-251 cells by RT-PCR using specific oligonucleotide primers. As shown in **Figure 3-5A (left)**, among the four receptor subtypes, ED-273b cells did not express EP1 receptor, whereas U-251 cells did not display EP1 and EP2 receptors. Both cell lines exhibited high levels of the EP4 receptor. Next, we employed a pharmacological approach to characterize the functional role of each receptor subtype in response to PGE_2-induced ERK1/2 and Akt phosphorylation. In this regard, ED-273b and U-251 cells were incubated with butaprost (a selective EP2 agonist), sulprostone (a selective EP3 agonist), PGE_1-OH (a selective EP4 agonist) or PGE_2 to determine their respective effects. As shown in **Figure 3-5A (right)**, PGE_1-OH stimulation caused a significant increase in ERK1/2 and Akt phosphorylation, an effect similar to that induced by PGE_2, and this effect was not seen in cells treated with butaprost and sulprostone. These data indicate that the EP4 receptor mediates the PGE_2-induced activation of ERK1/2 and Akt.

The specific functional role of the EP4 receptor in PGE_2-induced ERK1/2 and Akt phosphorylation was further examined by utilizing two approaches: pharmacological and molecular siRNA silencing of the EP4 receptor. First, we

treated ED-273b and U-251 cells with EP3 receptor antagonist ONO-AE3-240 and EP4 receptor antagonist ONO-AE3-208 to evaluate their respective effects. As shown in **Figure 3-5B-C**, inactivation of EP4 receptor with specific antagonist ONO-AE3-208 dose-dependently reduced the PGE_2-induced ERK1/2 and Akt phosphorylation in both cell lines. This effect was not seen with EP3 antagonists, thus providing pharmacological evidence for the EP4-mediated activation of ERK1/2 and Akt. Next, we silenced the expression of EP4 in ED-273b cells. Seventy two hours after transfection of these cells with EP4 small interfering RNA (*si*RNA), the cells were stimulated with either PGE_2 or butaprost to determine the degree of EP4 mediated ERK1/2 activation and analyzed by western blot. As predicted, EP4 receptor knockdown suppressed the expression of the EP4, whereas a control non-targeting siRNA (Lipo) had no effect (**Figure 3-5D**). Under these conditions, siRNA knockdown of the EP4 receptor markedly inhibited the PGE_2-induced phosphorylation of ERK1/2 but not by the butaprost (EP2 agonist), compared with the control group (Lipo) transfected with a control siRNA (**Figure 3-5D**). In addition, ED-273b cells transfected with EP4 siRNA completely suppressed the PGE_2-induced cell proliferation but was not affected by the butaprost treatment. Furthermore, this effect was not observed in cells transfected with a control siRNA (lipo) (**Figure 3-5E**). Taken together, these findings suggest that loss of EP4 function (whether through pharmacological inhibition or molecular receptor knockdown) significantly impacts on the PGE_2-induced ERK1/2 mitogenic activity.

PGE_2 Induces Multiple Downstream Targets

It has been demonstrated that EP4 stimulation by PGE_2 leads to increased expression of early growth response factor-1 (EGR-1) and T cell factor 4 (TCF-4) signaling through sequential activation of PI3K and ERK1/2 pathways (43, 44). Moreover, expression of microsomal prostaglandin E synthase-1 (mPGES-1) is linked with the increased PGE_2 production which promotes cell proliferation and growth in glioblastoma cells (45). For this reason, we sought to determine the ability of PGE_2 to induce several downstream targets such as EGR-1, TCF-4 and mPGES-1 in glioblastoma cells. Western blot analysis demonstrates that PGE_2 induced the expression of EGR-1, TCF-4 and mPGES-1 in a time-and-dose dependent manner (**Figure 3-6A**). To determine whether PGE_2-induced activation of PI3K signaling pathway is required for EGR-1 and COX-2 expression, we pretreated ED-273b cells with low dose 10 μM CXB overnight followed by rapamycin and various other mTOR/PI3K inhibitors, and analyzed the expression of EGR-1/COX-2 by western blot. Both PI3K inhibitors LY294002 and PI-103 (a dual PI3K and mTOR inhibitor) but not rapamycin (mTOR inhibitor) significantly inhibited the expression of EGR-1 and COX-2 and marginally decreased the expression of cyclin D1 (**Figure 3-6B**), a known target gene of TCF-4 (46), implicating the involvement of PI3K activity. In addition, PGE_2 or PGE_1-OH rescued the inhibitory effect of CXB on the expression of EGR-1/COX-2 along with cyclin D1, which is consistent with a previous report which showed that PGE_2 induced the expression of cyclin D1 (47) (**Figure 3-6B**). Furthermore, pretreatment of cells with ONO-AE3-208 (EP4 antagonist) in combination with

PGE_2 or PGE_1-OH significantly abolished the expression of EGR-1 and phosphorylation of ERK1/2 but not in cells pretreated with the selective EP2 agonist butaprost (**Figure 3-6C**). Our data indicate that the PGE_2-enhanced expression of EGR-1 requires the EP4 receptor and activation of the ERK1/2 pathway. Taken together, these findings suggest that PGE_2 exerts its actions through multiple downstream targets that are modulated by way of PI3K and ERK1/2 signaling to enhance EGR-1-mediated COX-2 expression possibly through an autocrine feedback loop.

PGE_2 Stimulates Cell Invasion via EP4-mediated Pathway

Several reports have demonstrated a functional role for Src as a mediator of cellular migration and cell proliferation via PGE_2-induced transactivation of the EGFR signaling in colon cancer (4, 39). The role of PGE_2 in GBM cells invasion has not been previously reported. To determine whether PGE_2-induced transactivation of EGFR results in increased invasiveness of GBM cells, we treated cells with PGE_2 alone or in the presence of various inhibitors and evaluated the effects of PGE_2-induced invasion on ED-273b cells grown in Matrigel®. As shown in **Figure 3-7**, PGE_2 treatment resulted in a 2-2.5 fold increase in Matrigel invasion, whereas pharmacologic antagonists of EGFR (AG; AG1478), Src (PP2) or EP4 (EP4 ant; ONO-AE3-208) inhibited ED-273b cells invasion through Matrigel inserts, compared with non-treated control. These data suggest that both PGE_2 and Src augment cell invasion through transactivation of the EGFR mediated by EP4 receptor.

3.4. Discussion

Several studies have shown that PGE_2 stimulation promotes tumor growth and cell migration through EP receptor-mediated transactivation of the EGFR via activation of the PI3K/Akt and/or the ERK1/2 pathways in colon cancer (4, 39) and cholangiocarcinoma (12). More recently, a study reported that COX-2-derived PGE_2 promotes medulloblastoma cell proliferation and growth via EP1 and EP3 receptors *in vitro* and *in* vivo (5). As yet, the role of PGE_2 in modulating the biological effects of primary glioblastoma cells has not been established. Here, to our knowledge, we present the first evidence that COX-2-derived PGE_2 promotes cell proliferation via the EP4 receptor and that PGE_2 transactivates EGFR and triggers activation of the PI3K/Akt and ERK1/2 signaling pathways. Furthermore, we demonstrate that PGE_2 induces phosphorylation of the EGFR via an intracellular Src-mediated pathway and that the interplay between EP4 and EGFR signaling enhances cell proliferation, invasion and tumor cell growth, as illustrated in **Figure 3-8**.

In line with the early reported *in situ* studies in human glioma tumors (9, 30), our data show that early passage of primary human glioblastoma cultures constitutively express COX-2 and secrete high amounts of PGE_2 which is correlated with the elevated endogenous COX-2 expression levels. These results support the relevance of previous findings in this study, suggesting a role for COX-2-derived PGE_2 in glioma progression. Although our early study (in Chapter 2) showed that the selective COX-2 inhibitor celecoxib blocked endogenous PGE_2

synthesis and induced the cytotoxic effect of glioblastoma cells only at the high concentrations (>25 μM), the precise mechanism of this effect was not evaluated. In this chapter we demonstrate that exogenously added PGE_2 restored cell proliferation of both celecoxib-treated ED-273b (COX-2 positive) and U-251 (COX-2 negative) cells. Notably, the growth-promoting effect of PGE_2 was more prominent in U-251 cells than that of ED-273b cells but surprisingly this effect was not seen in ED-273b cells in the absence of celecoxib treatment. The premise that exogenous PGE_2 restores (promotes) the growth of ED-273b cells to the levels in the absence of CXB treatment indicates the effects of COX-2 inhibition by CXB are in part mediated by the PGE_2. These results suggest that the endogenous PGE_2 levels in the ED-273b cells may play a role to support cell proliferation under the steady-state conditions. Interestingly, exogenous PGE_2 stimulation amplified the expression of COX-2 in a dose-dependent fashion in ED-273b cells but not in U-251 cells. The underlying mechanism is unknown; however, the PGE_2-induced COX-2 expression is likely regulated through a positive feedback loop, explaining why COX-2 is overexpressed in glioma tumors. Thus, our data indicate that COX-2-derived PGE_2 has a critical role in promoting cell proliferation and growth, thus underscoring PGE_2 as a potent mitogen.

There is considerable evidence to indicate that PGE_2 stimulates tumor cell proliferation and growth through a novel GPCR-EGFR receptor cross-talk mechanism (4, 12, 48, 49) and that the role of transactivation of the EGFR signaling by GPCR is commonly seen in many cell types (50, 51). Furthermore,

excessive EGFR signaling occurs invariably in gliomas (52, 53). In this study, we sought to determine the underlying molecular mechanism(s) by which COX-2-derived PGE_2 stimulates glioma cell proliferation and growth. Our results demonstrate the first evidence of PGE_2-induced cell proliferation via transactivation of the EGFR in glioblastoma ED-273b cells. This conclusion is based on the premise that PGE_2 induced EGFR phosphorylation and that the EGFR specific tyrosine kinase inhibitor AG1478 completely blocked the effect of PGE_2, thus inhibiting PGE_2-induced cell proliferation. These results are supported by others showing the presence of PGE_2-mediated transactivation of the EGFR in colon cancer and cholangiocarcinoma (4, 12, 39).

In addition, western blot analysis shows that PGE_2 upregulated the activity of the ERK1/2 and PI3K/Akt in a time-and dose-dependent fashion as demonstrated by the induced phosphorylation of both ERK1/2 and PI3K/Akt in ED-273b cells. Suppression of EGFR activity by the specific kinase inhibitor AG1478 strongly abolished both the ERK1/2 and Akt activation mediated by PGE_2 in numerous other glioma cell lines tested. Our results indicate a pivotal role for EGFR activation in GPCR-mediated stimulation of the ERK1/2 and Akt cascades and that PGE_2-induced EGFR transactivation by GPCR is a more general phenomenon in gliomas. However, we cannot rule out the possibility that GPCR stimulation (e.g. EP4) can also activate the ERK/MAPK cascade in the absence of EGFR via secondary messengers such as cAMP or G protein $\beta\gamma$ subunits and induce cellular proliferation (54).

PGE_2 exerts its diverse biological actions by binding to one of the four distinct GPCR (EP1-EP4) (55). EGFR transactivation mediated by GPCR agonists can occur via two distinct cellular signaling routes (56, 57). For the extracellular pathway, activated GPCR induces metalloproteinase-mediated release of EGF and EGF-like ligand(s) (58, 59) whereas the intracellular route involves the Src family of non-receptor tyrosine kinases and the reactive oxygen species-mediated inactivation of EGFR phosphatase (60, 61). Our study shows that PGE_2-induced transactivation of the EGFR occured through an intracellular mechanism via the activation of Src. Inhibition of Src activity with the pharmacologic antagonist PP2 completely abolished the activation of the EGFR, thereby inhibiting cell proliferation and growth and Matrigel invasion. This is not surprising as Src has been shown to directly phosphorylate and activate the EGFR pathway to promote tumor cell growth and progression in breast cancer (62). Furthermore, our data show that PGE_2 induced activation of Src in a dose-and-time dependent fashion as reflected by the phosphorylation of Src at Tyr-416, a marker for Src activation (63). Intriguingly, inhibition of EGFR with the specific kinase inhibitor AG 1478 had no observable effect on the ability of PGE_2 to phosphorylate and activate Src. This finding is consistent with the study in colon cancer showing that PGE_2-induced transactivation of EGFR occurs via an intracellular Src mechanism (39). Recent studies have shown that Src is frequently activated in glioblastoma *in vitro and in vivo (64)* and has been found to be an effector of the EGFR signaling in glioblastoma, resulting in enhanced GBM invasion and tumor cell survival (65). Our results are in agreement with

these findings, demonstrating the involvement of Src as a key mediator of the EGFR signaling which correlates with other reports showing an intracellular role of Src in PGE_2-induced transactivation of EGFR in colon cancer cells (4, 39) and in bile-duct cancer cells (12). The mechanism of GPCR-induced release of EGFR ligands via an extracellular route (58) was not investigated in our study, but remains an ongoing research initiative.

Although the precise role of each EP receptor in cancer initiation and progression including glioblastoma has yet to be fully characterized, individual EP receptor "knockout" mouse models have demonstrated that all four EP receptors have been implicated in promoting cancer cell growth and progression (66-68). For example, EP4 has been shown to be up-regulated in colon cancer (69) and in high grade gliomas (III and IV) (29) and appears to correlate with tumor grade and cell growth (29, 70). EP4 stimulation by PGE_2 induces colon carcinogenesis (71), mediates lung cancer invasiveness (72) and breast cancer cell metastasis (73) while EP2 and EP3 show opposing actions on cell growth (74). In this regard, our PCR analysis shows that not all the EP receptors were expressed in glioblastoma ED-273b and U-251 cells. For example, the expression profiles of EP2, EP3 and EP4 receptors were detected in ED-237b cells but only EP3 and EP4 were observed in U-251 cells. Of interest, we found that stimulation of PGE_2 or PGE_1-OH (EP4 agonist) in ED-273b and U-251 cells caused substantial increases in the phosphorylation of the ERK1/2 and Akt mitogenic pathways but not in cells treated with sulprostone (EP3 agonist) and butaprost (EP2 agonist), suggesting that the EP4 receptor plays a critical role in PGE_2-induced ERK1/2

and PI3K activities in gliomas. These observations are further corroborated by blockade with the selective EP4 antagonist ONO-AE3-208 which dose-dependently attenuated PGE_2-induced effects of ERK1/2 and Akt activation. Similarly, these effects were not seen in cells pretreated with EP3 antagonist (ONO-AE3-240). Due to the lack of commercially available EP2 antagonists, we are not able to investigate the effects of EP2 in these cells. In addition, silencing siRNA EP4 receptor study demonstrates that transfection of ED-273b cells with EP4 small interfering RNA (*si*RNA) suppressed the expression of the EP4 receptor and thereafter the PGE_2-induced ERK1/2 activation and cell proliferation. A similar transfection study performed in the U-251 cells was unsuccessful which may have been due to the high passage rate producing phenotypic changes of this cell line. However, our study provides convincing evidence that the activation of EP4 receptor by PGE_2 induces the ERK1/2 and Akt activities, leading to stimulation of glioblastoma cell proliferation.

Interestingly, the interplay between EGFR-Ras/ERK1/2 and PI3K/Akt signaling in glial progenitor cells induces GBM formation – mimicking human glioma tumors in the mouse (28) and the fruit fly (75), whereas neither EGFR/Ras nor Akt activity alone was sufficient to trigger human GBM in the mouse model (28). The combined Akt/ERK1/2 activity was correlated with high grade glioma generation which appeared to be linked to the glial progenitor cells (28). This is in contrast with early human GBM studies which frequently showed independent elevated Akt or ERK1/2 activities (27, 76). However, these findings are not supported by recent studies and our study supports the thesis that both the Akt and

ERK1/2 pathways are simultaneously activated by the EP4-mediated PGE_2 signaling, a feature critical for glioblastoma tumor growth and development.

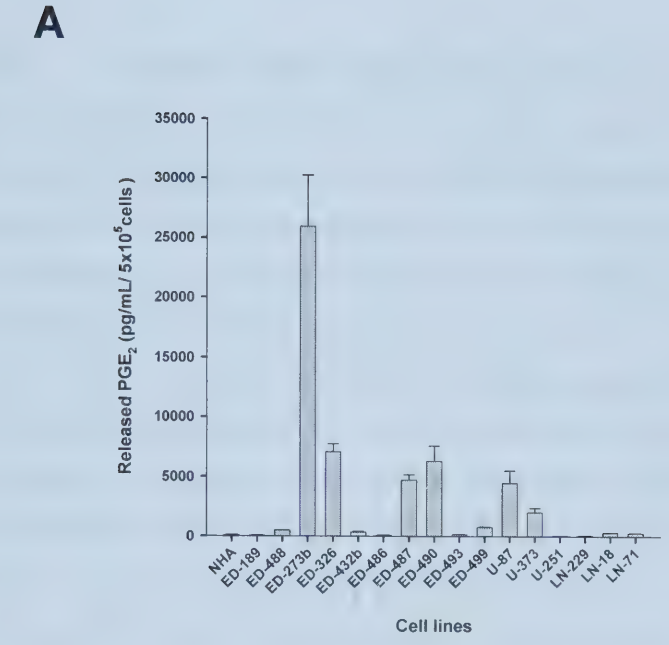
Our finding of PGE_2-induced EGR-1 expression is of particular interest. It is known that EGR-1 is a transcriptional factor which can be induced by growth factors such as EGF in GBM cells (77) and is regulated through Akt and ERK1/2 signaling pathways (46). In addition, EGR-1 has been shown to exert a dual role of tumor promoter and tumor suppressor function. Previous studies have shown that in tumor cell lines including astrocytoma, EGR-1 appeared to modulate cell proliferation, migration, invasion and angiogenesis (78-81). In contrast, EGR-1 was also perceived as a tumor suppressor gene rather than a transcription factor in breast (82), lung (82, 83) and surprisingly glioblastoma (84, 85). Nevertheless, the role of PGE_2 in EGR-1 expression has not been investigated. Our studies provide first evidence that PGE_2-induced activation of the EP4 receptor leads to EGR-1 expression through ERK1/2 phosphorylation via a PI3K-dependent pathway. This finding is in line with a study in colon cancer cells showing that PGE_2-induced activation of the EP4 receptor lead to EGR-1 expression through ERK1/2 phorphorylation in a PI3K-dependent pathway (43). Furthermore, we found that PGE_2 induced the activation of TCF-4 and cyclin D1 expression, consistent with a study demonstrated by others (44). Interestingly, PGE_2 synthase is up-regulated by EGR-1 (86); cyclin D1 is a target of EGR-1 and is upregulated via both PI3K and ERK1/2 pathways (87). Thus, EGR-1 acts downstream of EP4 signaling to increase PGE_2 synthase through a PGE_2-mediated induction of EGR-1 positive

feedback loop in glioblastoma cells. However, further studies are needed to clarify these findings.

In summary, we show for the first time that EP4 receptor plays a pivotal role in PGE_2-induced transactivation of EGFR signaling to promote cell proliferation, growth and invasion in glioblastoma cells. Our findings also support the premise that COX-2-derived PGE_2 activates ERK1/2 and PI3K/Akt signaling pathways, which result in further amplification of COX-2 expression, thus enhancing EGFR signaling via activation of the EP4 receptor. The molecular cross-talk between the COX-2-derived PGE_2 and EGFR signaling may be important in maintaining COX-2 overexpression in glioma tumor cells. Given the reported significant side effects of selective COX-2 inhibitors and poor treatment efficacy in GBM, our findings suggest that targeting both the EP4 receptor and EGFR signaling network may be an efficacious therapeutic combination strategy to prolong quality patient survival.

Figure 3-1. Early explant glioblastoma cells express elevated levels of COX-2 and PGE_2. A, a panel of 17 primary and secondary GBM cell lines were seeded at a concentration of 5 x 10^5 cells/well in 6-well plates. After 24 h incubation, the culture medium were collected and subjected to PGE_2 measurement by EIA according to the manufacturer's protocol. **B**, the representative cell lysates (50 μg) were immunoblotted with anti-COX-2 antibody.

Figure 3-1.


A
35000
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Released PGE2 (pg/mL/ 5x10^6 cells)
NHA
ED-189
ED-488
ED-273b
ED-326
ED-432b
ED-486
ED-487
ED-490
ED-493
ED-499
U-87
U-373
U-251
LN-229
LN-18
LN-71
Cell lines

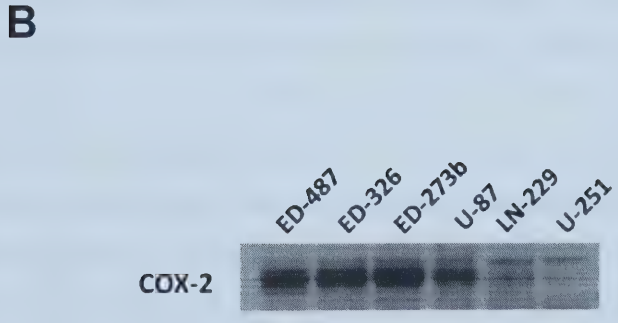

B
ED-487
ED-326
ED-273b
U-87
LN-229
U-251
COX-2

Figure 3-2. Celecoxib inhibits GBM cell proliferation, which can be rescued by exogenously added PGE_2. **A**, ED-273b and U-251 cells (5 x 10^3cells/well) seeded in 96-well plates were treated with increasing concentrations of celecoxib (1.56-100 μM) in the presence of 0.5% FCS for 24 h. Dose-response curve was established by measuring absorbance of the formazan dye using a XTT cytotoxic assay kit. Results shown were plotted as the percentage (%) cytotoxicity mean ± SEM of three triplicates wells of three experiments standardized to control untreated cells. **B**, ED-273b and U-251 cells were untreated (-CXB) or pretreated with a low dose of 10 μM celecoxib (+CXB) in the presence of 0.5% FCS for 24 h prior to treatment of various doses of PGE_2 (0.63 - 2.5 μM) for 15 min in the fresh medium for 3 days (replenished daily). On the 4th day, 2 μCi of methyl-[^{3}H] thymidine was added to each well and the plates were incubated for another 4 h. Cell proliferation was measured as incorporated [^{3}H] thymidine in cpm (count per minute). Results shown were plotted as the averages ± SEM (*, $p<0.05$ vs. untreated COX inhibitor; **, $p<0.05$ vs. treated COX inhibitor without PGE_2) of three experiments. **C**, cell proliferation induced by PGE_2 was evaluated as described under "Materials and Methods." Cell numbers were counted manually with a haemocytometer and were averaged from triplicate wells. Results shown were plotted as the averaged ± SEM (*, $p< 0.05$ vs. control; **$p<0.05$ vs. celebrex (CXB). **D**, cell lysates (50 μg) of ED-273b (**top**) and U-251 (**bottom**) were immunoblotted with anti-COX-2 antibody and anti-ERK1/2 antibody for total protein loading controls.

Figure 3-2

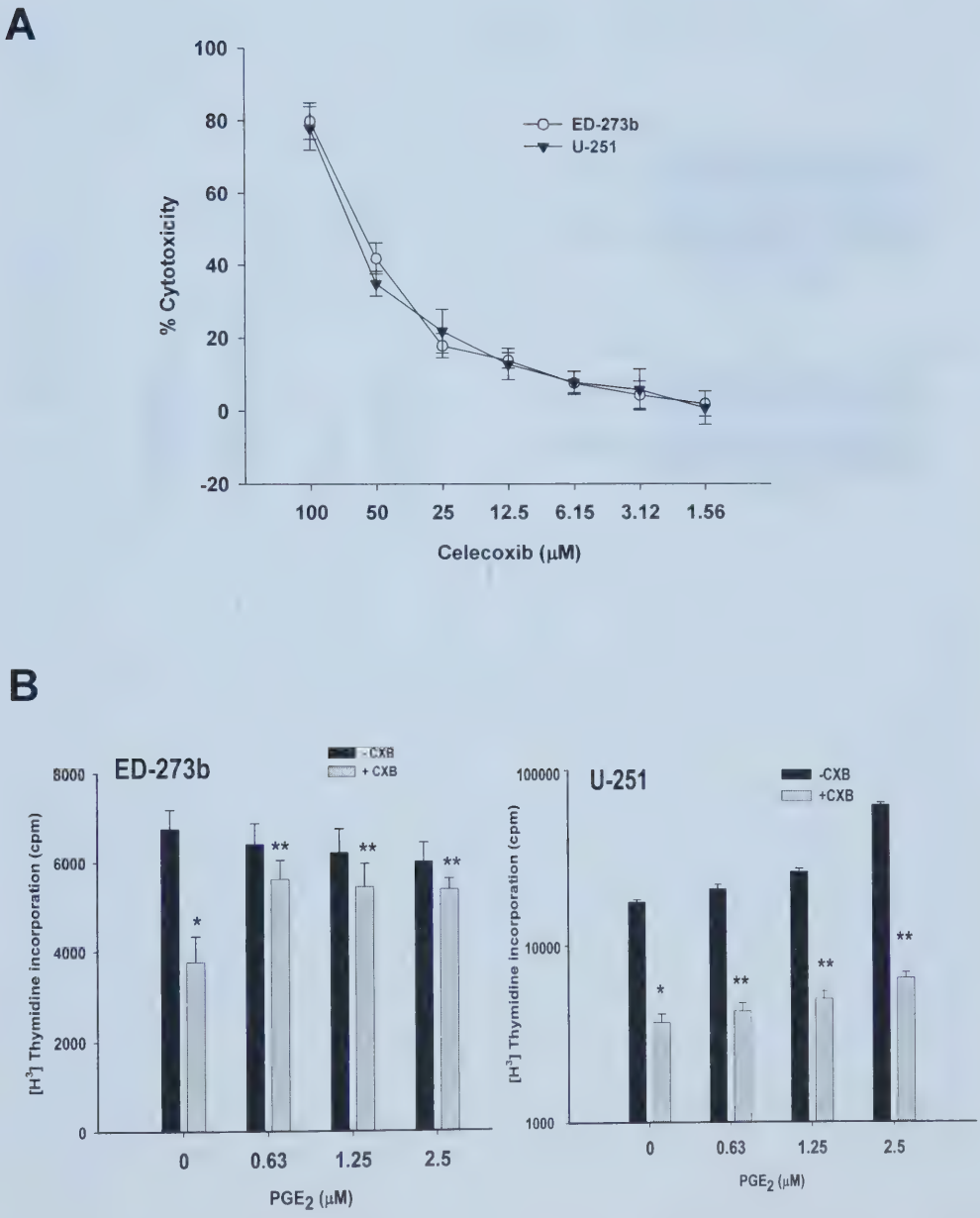

A
100
80
60
40
20
0
-20
% Cytotoxicity
ED-273b
U-251
100
50
25
12.5
6.15
3.12
1.56
Celecoxib (μM)
B
ED-273b
-CXB
+CXB
8000
6000
4000
2000
0
[H3] Thymidine incorporation (cpm)
*
**
**
**
0
0.63
1.25
2.5
PGE2 (μM)
U-251
-CXB
+CXB
100000
10000
1000
[H3] Thymidine incorporation (cpm)
*
**
**
**
0
0.63
1.25
2.5
PGE2 (μM)

Figure 3-2

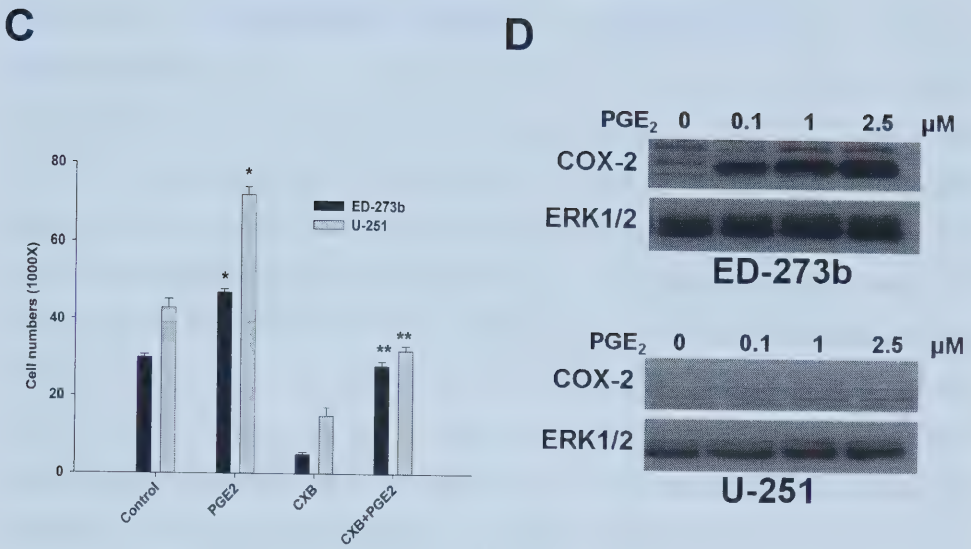

C
D
Cell numbers (1000X)
80
60
40
20
0
ED-273b
U-251
*
*
**
**
Control
PGE2
CXB
CXB+PGE2
PGE2 0 0.1 1 2.5 μM
COX-2
ERK1/2
ED-273b
PGE2 0 0.1 1 2.5 μM
COX-2
ERK1/2
U-251

Figure 3-3. PGE_2-induced activation of the ERK1/2 and Akt involves EGFR signaling. **A**, serum-starved ED-273b cells were stimulated with PGE_2 for the indicated times (**left panel**) or 50 ng/EGF, no treatment, 2.5 μM PGE_2 alone or in combination of pretreatment of 10 μM AG 1478 (AG, 1 h) (**right panel**). The cell lysates were immunoprecipitated with anti-EGFR antibody and the immunoprecipitates were immunoblotted with tyrosine-phosphorylated (PY 100) antibody. The blots were probed with anti-EGFR antibody for total protein loading controls. **B**, serum-starved ED-273b cells were stimulated with various doses of PGE_2 (**left panel**) and 2.5 μM PGE_2 for periods of time, as indicated (**right panel**), and analysed by western blot for PGE_2-induced ERK1/2 and Akt activation using anti-phospho-ERK1/2 (pERK1/2) and anti-phospho-Akt (pAkt) antibodies. The same blots were stripped and reprobed with anti-ERK1/2 or anti-Akt antibodies for total protein loading controls. **C**, primary cultures of ED-273b and ED-189 and long-term established LN-229 and U-251 cell lines were serum-starved for 24 h and treated with EGFR-specific inhibitor 10 μM AG1478 or untreated for 60 min prior to 2.5 μM PGE_2 stimulation for 15 min. The cell lysates were immunoblotted with pERK1/2 and pAkt antibodies and the same blots were reprobed with anti-ERK1/2 or Akt antibodies for total protein loading controls. **D**, ED-273b and U-251 cells were treated with 2.5 μM PGE_2 alone, or pretreated with 10 μM AG1478 (AG) for 1 h followed by PGE_2 for 15 min. Cell proliferation was measured by [^{3}H] thymidine incorporation assay. Results shown were plotted as the average ± SEM (*, $p<0.05$ vs. control; **, $p<0.05$ vs. PGE_2) of three triplicates wells of three independent experiments.

Figure 3-3

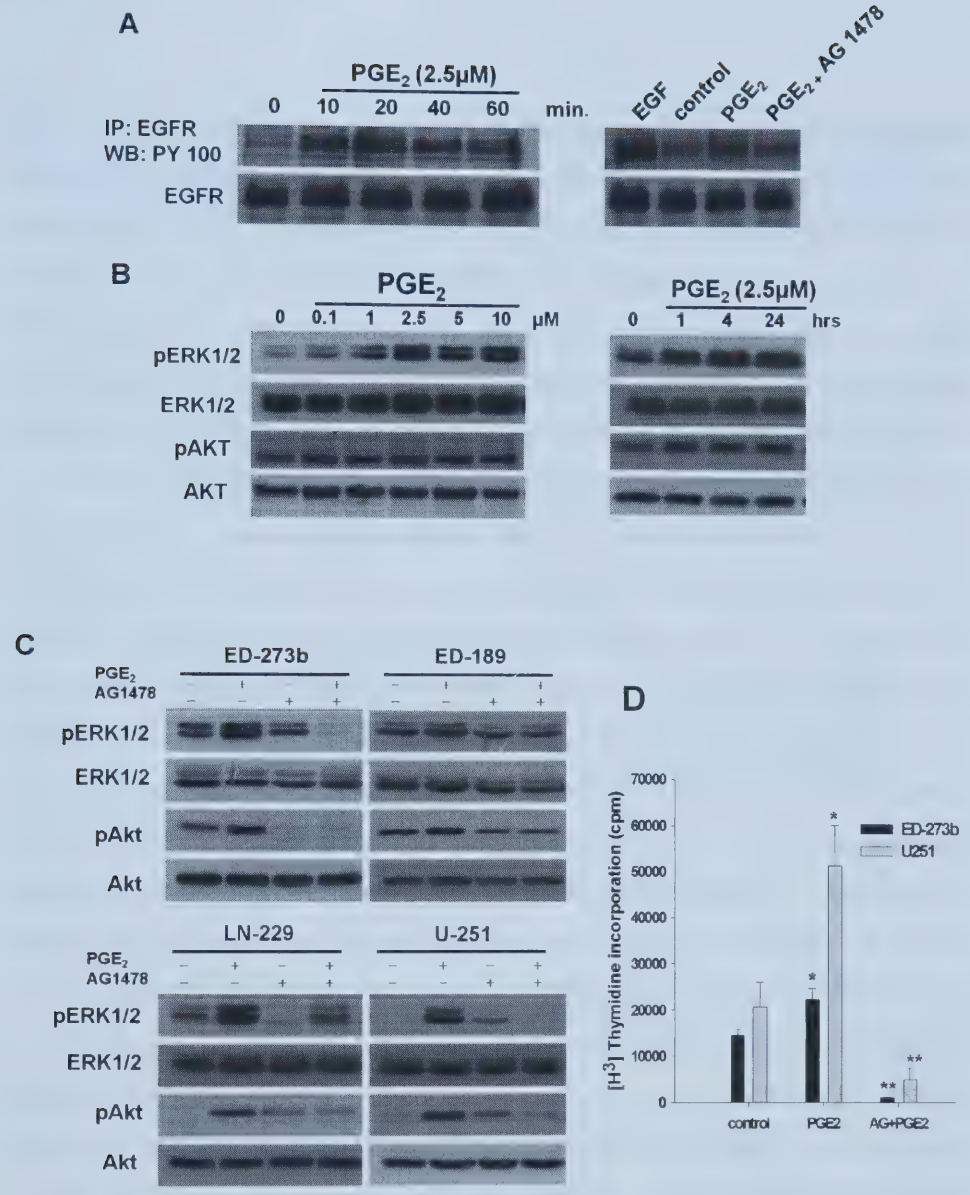

A
PGE2 (2.5μM)
0 10 20 40 60 min.
IP: EGFR
WB: PY 100
EGFR
EGF
control
PGE2
PGE2 + AG 1478
B
PGE2
0 0.1 1 2.5 5 10 μM
PGE2 (2.5μM)
0 1 4 24 hrs
pERK1/2
ERK1/2
pAKT
AKT
C
ED-273b
ED-189
PGE2
AG1478
pERK1/2
ERK1/2
pAkt
Akt
LN-229
U-251
D
[H3] Thymidine incorporation (cpm)
70000
60000
50000
40000
30000
20000
10000
0
ED-273b
U251
control
PGE2
AG+PGE2

Figure 3-4. PGE_2 transactivates EGFR via an intracellular Src kinase mechanism. A, (upper panel) serum-starved ED-273b cells were stimulated with various doses (0.1- 10 μM) of PGE_2 for 15 min (**left**) and 2.5 μM PGE_2 for periods of time, as indicated (**right**). The cell lysates were immunoblotted for PGE_2-induced Src activation analysis using anti-pSrc antibody. The same blots were stripped and reprobed for Src to evaluate protein loading. **A**, (**lower panel**) serum-starved ED-273b cells were pretreated with Src kinase inhibitor PP2 (0.1, 1, 5, 10 μM, 1 h), prior to 2.5 μM PGE_2 stimulation for 15 min. The cell lysates were subjected to western blot analysis. The same blot was reprobed for ERK1/2 to evaluate protein loading. **B**, serum-starved ED-273b cells were lysed after 2.5 μM PGE_2 stimulation with the indicated times (**upper panel**), PGE_2 alone, or in the presence of 10 μM PP2 (**lower panel**) and were immunoprecipitated with anti-Src antibody or anti-EGFR antibody, respectively. The immunoprecipitates were immunoblotted with either anti-pEGFR antibody followed by reprobing with anti-Src antibody (**upper panel**) or tyrosine-phosphorylated (PY 100) antibody followed by reprobing with anti-EGFR antibody (**lower panel**). **C**, western blot analysis of PGE_2-induced Src activation in the presence or absence of EGFR-specific inhibitor AG 1478 (**left panel**) and PP2 (**right panel**). **D**, ED-273b and U-251 cells (1x 10^5cells/well) in 24-well plates were serum-starved overnight and untreated (control), treated with 2.5 μM PGE_2 or in combination of ERK1/2 inhibitor PD98059 (PD, 25 μM) or PP2 (10 μM). Cell proliferation was measured by [^{3}H] thymidine incorporation and cell numbers were counted and averaged from triplicate wells standardized to cell count. Results shown were plotted as the averages ± SEM (*, $p < 0.05$ vs. control) of three independent experiments.

Figure 3-4.

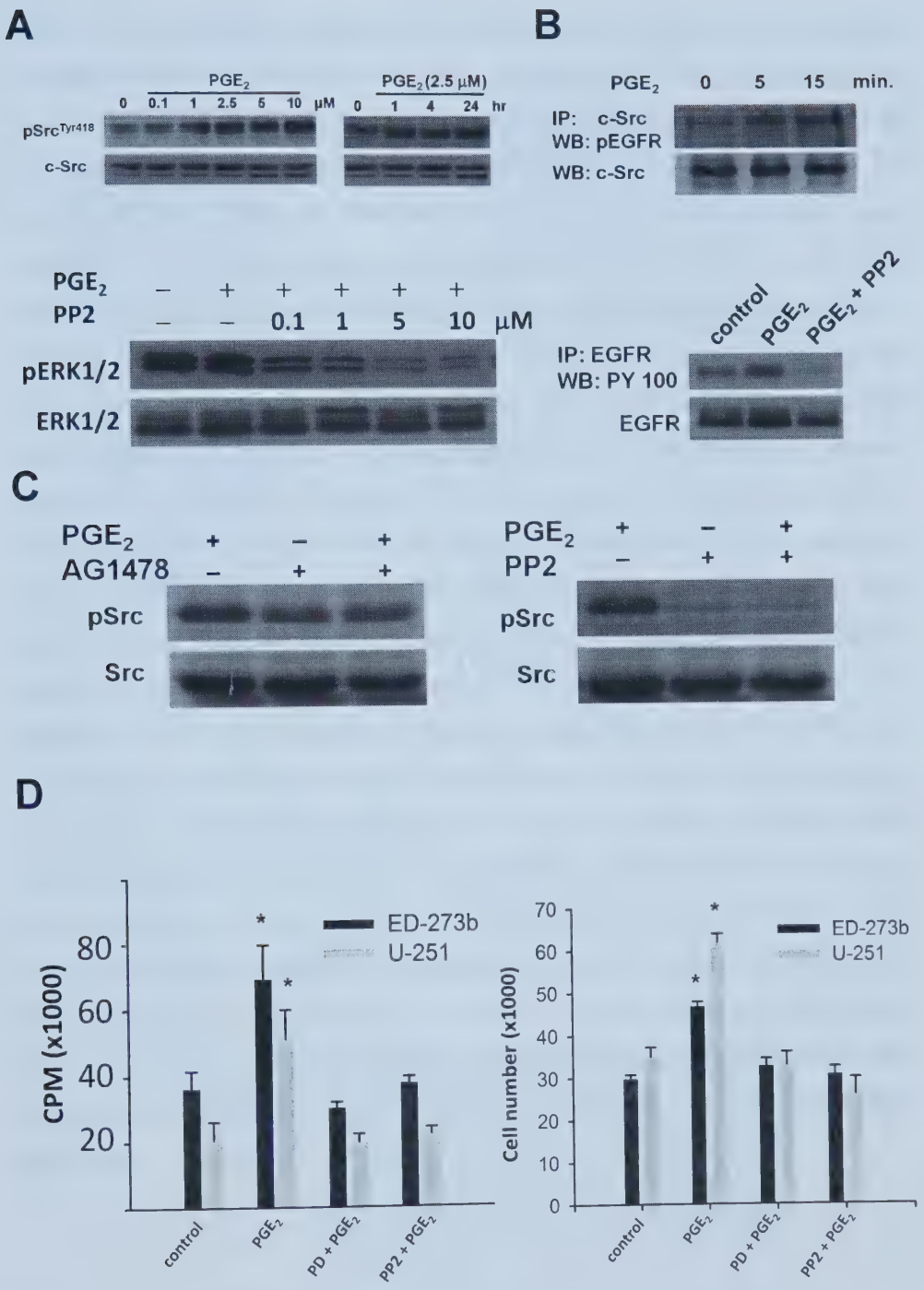

A
PGE2
0 0.1 1 2.5 5 10 μM
PGE2 (2.5 μM)
0 1 4 24 hr
pSrcTyr418
c-Src
PGE2 – + + + + +
PP2 – – 0.1 1 5 10 μM
pERK1/2
ERK1/2
B
PGE2 0 5 15 min.
IP: c-Src
WB: pEGFR
WB: c-Src
control
PGE2
PGE2 + PP2
IP: EGFR
WB: PY 100
EGFR
C
PGE2 + – +
AG1478 – + +
pSrc
Src
PGE2 + – +
PP2 – + +
pSrc
Src
D
CPM (x1000)
80
60
40
20
ED-273b
U-251
control
PGE2
PD + PGE2
PP2 + PGE2
Cell number (x1000)
70
60
50
40
30
20
10
0
ED-273b
U-251
control
PGE2
PD + PGE2
PP2 + PGE2

Figure 3-5. PGE_2-induced ERK1/2 and Akt activation involves EP4 receptor. **A**, (**left panel**) RT-PCR analysis of PGE_2 receptors (EP1-EP4) expression. One μg of total RNA isolated from ED-273b and U-251 cells was reverse-transcribed with use of Superscript III transcriptase and amplified using primers specific for human EP1, EP2, EP3 and EP4 for 35 cycles. The amplified products were visualized on 1.2% agarose gels. **A**, (**right panel**) ED-273b and U-251 cells were serum-starved for 24 h and untreated (control) or treated with 2.5 μM PGE_2, 2.5 μM PGE_1-OH, 10 μM sulprostone, 5 μM butaprost for 15 min. The cell lysates were immunoblotted with anti-pERK1/2 and pAkt antibodies. The same blots were stripped and reprobed with anti-ERK1/2 and Akt antibodies for protein loading controls. **B** (ED-273b) and **C** (U-251), the cells were serum-starved for 24 h and treated with 2.5 μM PGE_2, increasing concentrations of EP4 antagonist ONO-AE3-208 (0.1, 1, 5, 10 μM), EP3 antagonist ONO-AE3-240 (10 μM) or in combination of PGE_2 for 15 min, as indicate. The cell lysates were immunblotted against anti-pERK1/2 and pAkt antibodies. The same blots were reprobed with anti-ERK1/2 and Akt antibodies for protein loading controls. **D**, ED-273b cells were transiently transfected with EP4 *SMART*pool *si*RNAs or with Lipofectamine containing a control non-targeting *si*RNA (Lipo). Seventy two hours after transfection, the cells were treated 2.5 μM PGE_2 or 5 μM butaprost for 15 min. Gene knockdown of the expression of EP4 and phosphorylation of ERK1/2 were analyzed by western blot. **E**, cell proliferation of ED-273b cells transfected with EP4 *SMART*pool *si*RNAs (*si*RNA) or Lipofectamine containing a control *si*RNA (lipo) was measured by [^{3}H] thymidine incorporation (cpm). Results shown were plotted as the average ±SEM (*<0.05 vs. control) of two independent experiments.

Figure 3-5.

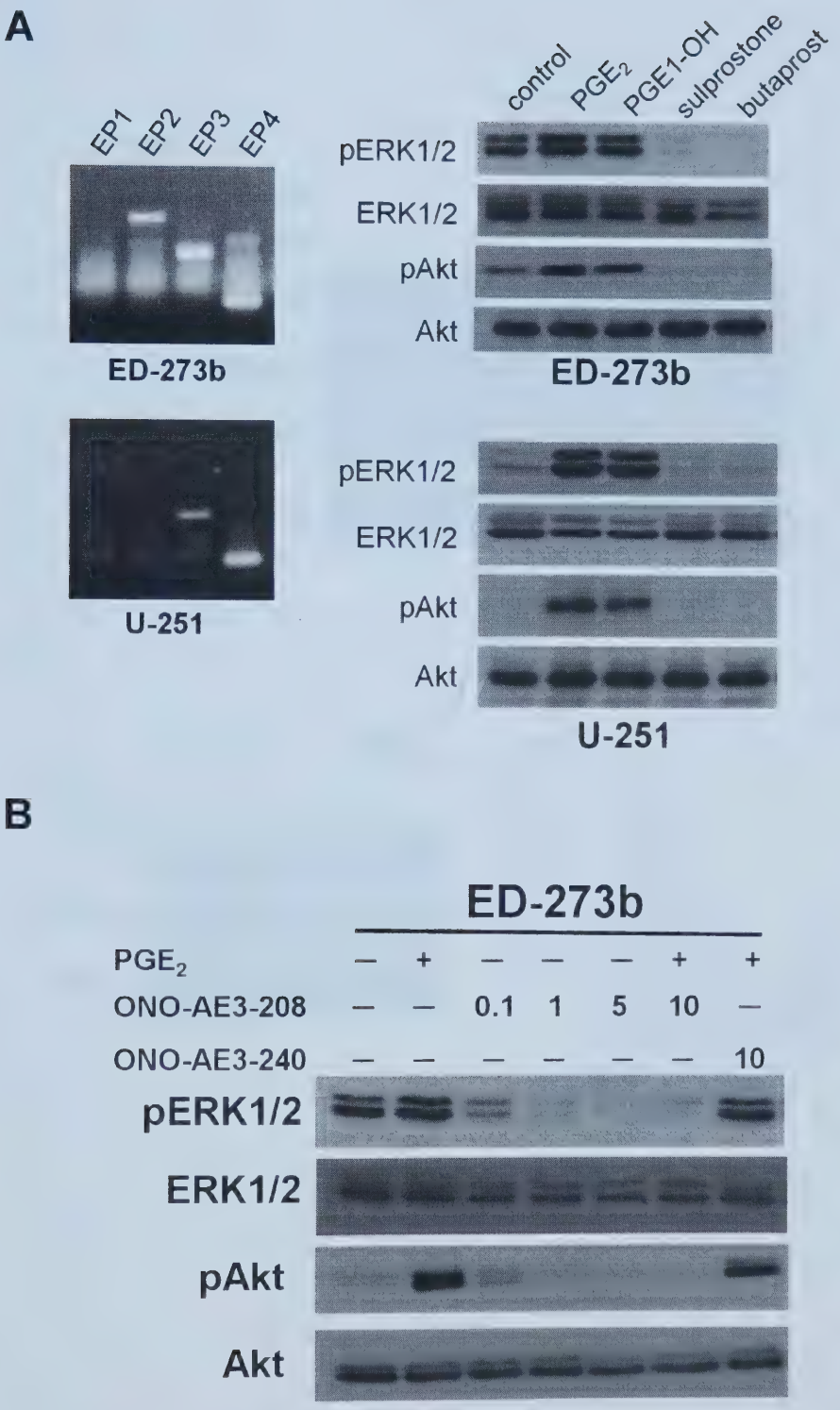

A
EP1
EP2
EP3
EP4
ED-273b
U-251
control
PGE2
PGE1-OH
sulprostone
butaprost
pERK1/2
ERK1/2
pAkt
Akt
ED-273b
pERK1/2
ERK1/2
pAkt
Akt
U-251
B
ED-273b
PGE2 — + — — — + +
ONO-AE3-208 — — 0.1 1 5 10 —
ONO-AE3-240 — — — — — — 10
pERK1/2
ERK1/2
pAkt
Akt

Figure 3-5.

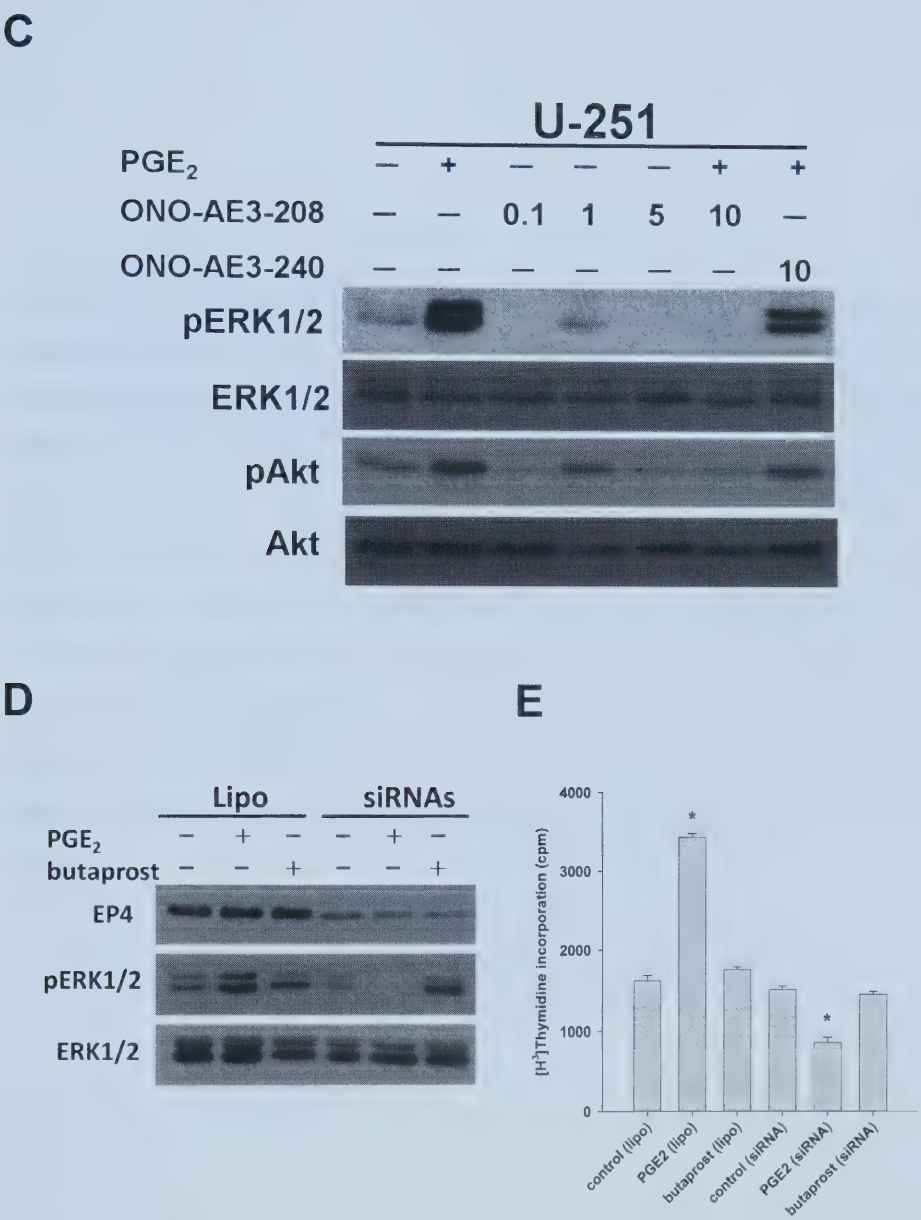
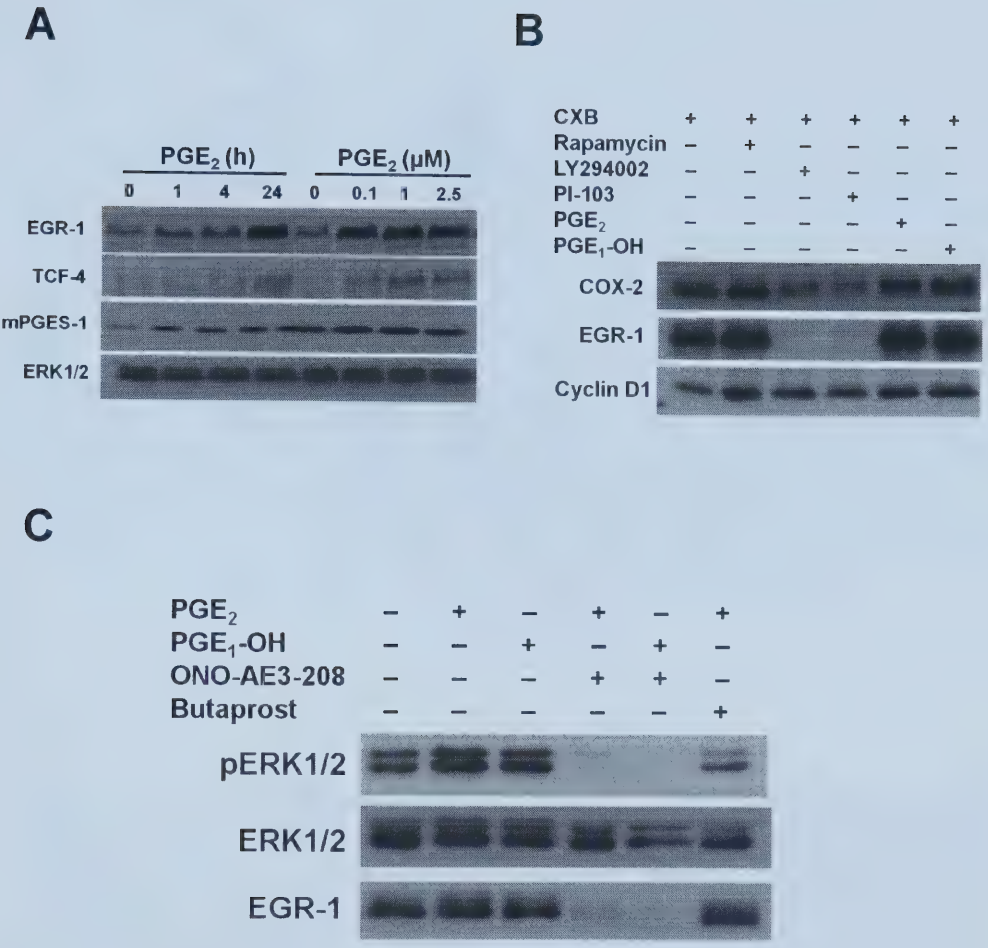

C
U-251
PGE2
ONO-AE3-208
ONO-AE3-240
0.1
1
5
10
pERK1/2
ERK1/2
pAkt
Akt
D
Lipo
siRNAs
PGE2
butaprost
EP4
pERK1/2
ERK1/2
E
[H3]Thymidine incorporation (cpm)
4000
3000
2000
1000
0
control (lipo)
PGE2 (lipo)
butaprost (lipo)
control (siRNA)
PGE2 (siRNA)
butaprost (siRNA)

Figure 3-6. PGE_2 activates multiple downstream targets and promotes cell invasion. **A**, serum-starved ED-273b were stimulated with 2.5 μM PGE_2 for the indicated times and incubated with various doses of PGE_2 (0.1, 1, 2.5 μM) for 4 h before western blot analysis using antibodies against EGR-1, TCF-4, mPGES-1 or total ERK1/2 protein. **B**, serum-starved ED-273b cells were pretreated with a low dose of 10 μM CXB followed by the addition of 100 nM rapamycin, 30 μM LY294002, 1 μM PI-103, 2.5 μM PGE_1-OH, or 2.5 μM PGE_2, and incubation for 4 h before immunoblot assays using antibodies against COX-2, EGR-1 or cyclin D1. **C**, serum-starved ED-273b cells were pretreated with ONO-AE3-208 or butaprost for 4 h followed by PGE_2 or PGE_1-OH stimulation for another 4 h before western blot analysis using antibodies against pERK1/2, ERK1/2 and EGR-1.

Figure 3-6.


A
PGE2 (h)
PGE2 (μM)
0 1 4 24 0 0.1 1 2.5
EGR-1
TCF-4
mPGES-1
ERK1/2
B
CXB + + + + + +
Rapamycin – + – – – –
LY294002 – – + – – –
PI-103 – – – + – –
PGE2 – – – – + –
PGE1-OH – – – – – +
COX-2
EGR-1
Cyclin D1
C
PGE2 – + – + – +
PGE1-OH – – + – + –
ONO-AE3-208 – – – + + –
Butaprost – – – – – +
pERK1/2
ERK1/2
EGR-1

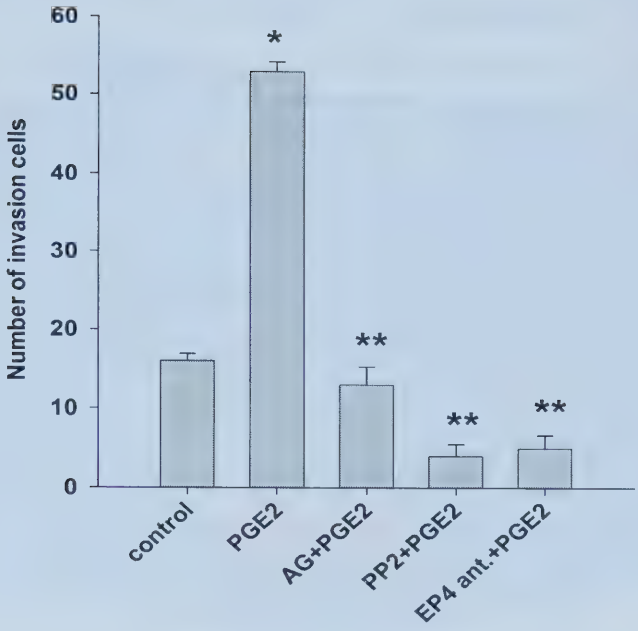


Figure 3-7. PGE_2 stimulates glioblastoma cell invasion via EP4-mediated pathway. Serum-starved ED-273b cells were seeded onto Matrigel® coated inserts with 8 μm pore size and the lower chamber was filled with media control, 2.5 μM PGE_2 alone, or in the presence of 10 μM AG1478 (AG), 10 μM PP_2, or 1 μM EP4 antagonist ONO-AE3-208 (EP4 ant). After 24 h incubation, the migrated cells in the bottom of the insert were fixed, stained, counted and averaged from three wells and standardized to cell count. Results shown were plotted as the average ± SEM (*, $p<0.05$ vs. control; **, $p<0.05$ vs. PGE_2) of two independent experiments.

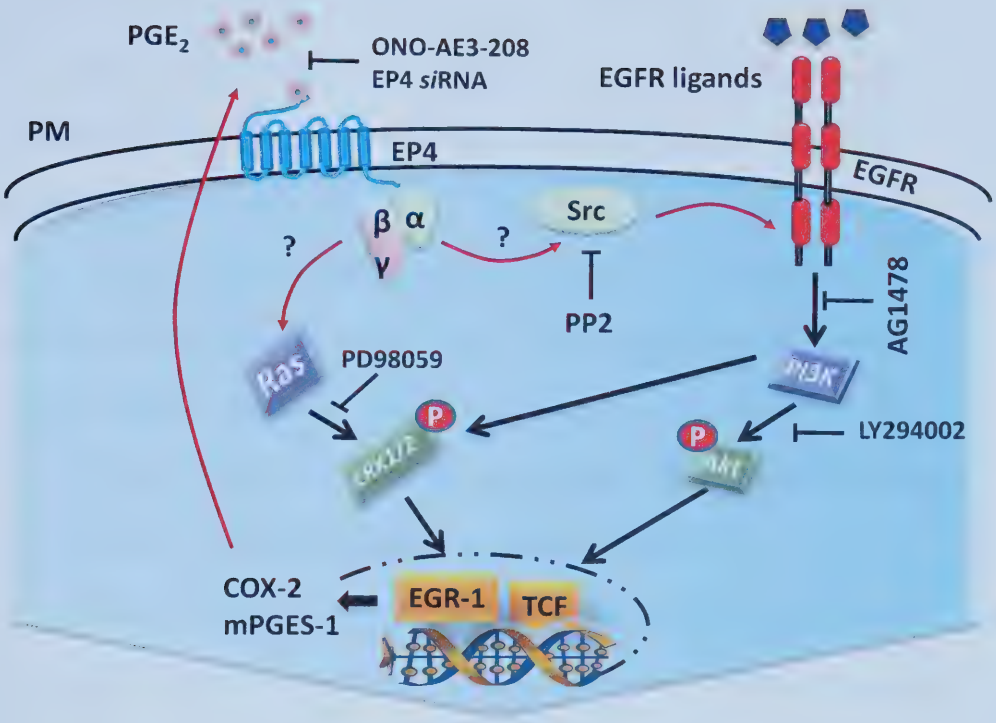


Figure 3-8. Schematic model for PGE_2-mediated EP4 receptor signaling in glioblastoma. PGE_2 transactivates the EGFR signaling via the EP4 receptor cross-talk by an intracellular Src mechanism, thereby activating ERK1/2 and PI3K/Akt. The activation of these signaling cascades is involved in growth and invasion of glioblastoma cells through transcription of EGR-1 and TCF which leads to upregulation of COX-2 and mPGES-1, thus providing a positive feedback loop for PGE_2. These signaling pathways could be blocked by specific EGFR kinase inhibitor AG1478, MEK kinase inhibitor PD98059, PI3K kinase inhibitor LY294002, Src inhibitor PP2, EP4 antagonist ONO-AE3-208 or siRNA against EP4.

3.5. References

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glioblastoma multiforme. Journal of the National Cancer Institute 2001;93(16):1246-56.

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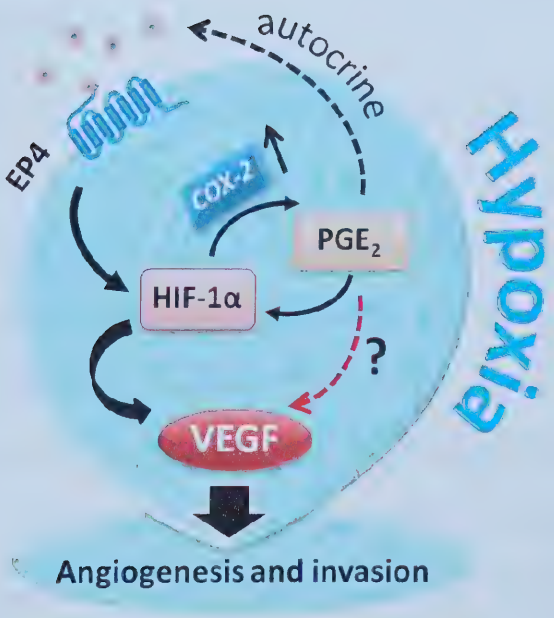
Chapter 4

PGE_2 Mediates HIF-1α Up-Regulation and Stimulates VEGF Production in Glioblastoma Cells



4.1. Introduction

Glioblastoma multiforme (GBM) is characterized by exuberant angiogenesis, a crucial event for brain tumor growth initiation, tumor progression and diffuse infiltrative invasion into surrounding brain tissue (1). However, several clinical antiangiogenesis studies have reported limited therapeutic efficacy of angiogenesis inhibitors in controlling tumor growth and progression (2-5). Recent studies utilizing a mouse model have reported that tumors spread faster and at a greater frequency to both nearby tissues and distant organs in mice treated with angiogenesis inhibitors (6, 7). These results have been explained by earlier studies indicating that hypoxia induced by antiangiogenesis treatment can enhance tumor cell motility and invasiveness. These findings also suggest that aggressive tumors possess multiple angiogenesis factors and pathways apart from the VEGF/VEGFR cascade (8).

GBM angiogenesis is a complex process initiated primarily by hypoxia which is largely mediated by hypoxia-inducible factors (HIF-1α and/or 2α) (9-12). Briefly, HIF-1 is a heterodimer transcriptional factor consisting of an oxygen-sensitive HIF-1α and a constitutively expressed HIF-1β subunit. HIF-1α has a short half-life of < 5 min *in vitro* and *in vivo* (13, 14). During normoxia, HIF-1α is ubiquitinated through prolyl hydroxylase 3 (PHD3)-mediated interaction with the von Hippel-Lindau (VHL) tumor suppressor protein (15) and thereafter targeted for proteasomal degradation (16, 17). Under hypoxia (0.5–2% O_2), HIF-1α is stabilized and translocates to the nucleus, heterodimerizing with HIF-1β (also

called aryl hydrocarbon receptor nuclear translocator, ARNT). This dimer binds to a hypoxia-responsive element (HRE) within DNA promoter regions of the target genes (18), thereby activating transcription of >100 target genes (19, 20). Targeted protein products are involved in cell survival, metabolism, angiogenesis, enhanced motility and metastasis (21, 22). Three HIF-α proteins have been discovered in which HIF-1α and HIF-2α are highly structurally related and bind to the same HRE sequences whereas HIF-3α lacks a transcriptional activation domain. HIF-1α is more universally expressed; HIF-2α is expressed in a more restricted pattern within normal tissues and in tumors with an aggressive phenotype (23) while HIF-3α functions as a negative regulator in response to hypoxia (24-27).

Although somatic mutations of p53 have been frequently found (i.e. >50%) in most cancers including GBMs (28), unlike, renal cell carcinomas, GBM tumors do not display somatic mutations of the VHL tumor suppressor gene (29). HIF-1α is overexpressed in glioma tumor specimens and the level of expression correlates with the highest grade of malignancy (30). In addition, HIF-1α is highly expressed in hypoxic pseudopalisading cells surrounding the necrotic zones, implicating that HIF-1α in GBM correlates with intra-tumoral hypoxia (31). Furthermore, *VEGF*, a target gene of HIF-1α and a potent stimulant of GBM angiogenesis, is up-regulated in hypoxic pseudopalisading cells near necrotic foci at both the mRNA and protein levels (30-32). Notably, VEGF expression is also regulated by growth factors and cytokines such as epidermal growth factor (EGF), insulin, insulin-like growth factor-I (IGF-I) and tumor necrosis factor α (TNF-α) and is modulated by

activity of the phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1 and 2 (ERK1/2) (33-37).

Hypoxia also potently triggers cyclooxygenase-2 (COX-2) transcription in various types of cancers (38-41). PGE_2, a metabolite of COX-2, is also a strong inducer of VEGF (42) and is upregulated by hypoxia whereas the COX-2 inhibitor NS-398 inhibits the induction of VEGF (39, 43). Thus, it is surmised that hypoxia modulates the COX-2 transcriptional target, thereby increasing PGE_2 levels leading to increased VEGF production via a novel positive feedback loop. In fact, previous studies have shown that PGE_2 mediates the hypoxic effects of VEGF by modulating HIF-1α expression in colon (39, 42) and prostate (44) cancers. However, the mechanism(s) by which PGE_2 induces VEGF expression in GBM has not been explored. In our study (Chapter 3), we showed that cultured GBM cells produce a significant amount of PGE_2. With this finding, we hypothesized that PGE_2 enhances hypoxia-mediated expression of HIF-1α, thereby increasing VEGF production in GBM.

4.2. Materials and Methods

Cell Culture and Reagents

Early passage (<10 passages) primary cultures of human glioma ED-273b derived from a surgical tumor specimen was established as previously described

(45). The long-term culture of the U-251 cell line was a generous gift from Dr. Charles Hao (Emory University School of Medicine, Atlanta, GA). Both cell lines were maintained in Dulbecco's modified Eagle's medium and F-12 nutrition mixture (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin and streptomycin and cultured under normoxic conditions (21% O_2, 5% CO_2, 75% N_2) in a humidified incubator at 37°C. In each experiment setting, cells were plated overnight and serum-starved for 24 h prior to drug treatment or hypoxic stimulation in a humidified hypoxic chamber (BioSpherix Ltd, Redfield, NY) flushed with 1% O_2 balanced with 5% CO_2 and 94% N_2 and sealed. 16,16-dimethyl PGE_2 (Cayman Chemical, Ann Arbor, MI) supplied in methyl acetate was evaporated with a flow of nitrogen air stream and immediately dissolved in a designated volume of 100% ethanol as required. Cobalt chloride and cycloheximide (CHX) were purchased from Sigma-Aldrich (St. Louis, MO). The kinase inhibitors such as PP2 (Src kinase) and PD98059 (extracellular signal-regulated kinase kinase 1 (MEK1) were purchased from Calbiochem Inc. (La Jolla, CA). EP4 receptor antagonist ONO-AE3-208 was a generous gift from ONO Pharmaceutical Co. Ltd. (Chuoku, Osaka, Japan). PI3-kinase inhibitor LY294002 and EP4 agonist PGE_1-OH were purchased from Cayman Chemical (Ann Arbor, MI). Unless otherwise indicated, all other chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada) and dissolved in dimethyl sulfoxide (DMSO).

Measurement of PGE_2 and VEGF Release

ED-273b and U-251 glioma cells (1x 10^6cells/well) were seeded onto 12-well plates overnight to attain 75-80% confluence. Cells were subjected to hypoxia treatment alone or in the presence of PGE_2 with or without celecoxib. After treatment, the culture supernatants were collected, centrifuged at 2,500 rpm for 5 min and used immediately or were frozen at -80°C for further analysis. The level of PGE_2 was measured using a PGE_2 EIA kit (Cayman Chemicals, Ann Arbor, MI) and VEGF was quantified using a Quantikine ELISA assay kit (R& D systems, Minneapolis, MN) according to the manufacturer's instructions.

Western Blotting Analysis

Experimental cell culture dishes were rinsed in PBS and cells collected as pellets in 1.5 mL eppendorf tubes and frozen at -80°C until further use. For cytosolic and nuclear fraction isolations, cell pellets were lysed using a commercial NE-PER® Nuclear and Cytoplasmic Extraction Reagents kit (Pierce, Rockford, IL) according manufacturer's instructions. Protein concentration was determined using modified Bradford protein assay kit (Pierce, Rockford, IL). The samples containing equal amounts of protein (30-50 µg) were resolved on 8 - 12% SDS-PAGE (dependent on the molecular weight of the proteins to be detected), and transferred to a 0.45µm nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked in 5% BLOTTO buffer [5% non-fat dry milk in TTBS (20 mM Tris-HCL, pH 7.5 + 150 mM NaCl + 0.1% Tween 20)]

for 1 h and then incubated overnight at 4°C with the following primary antibodies: anti-HIF-1α, anti-β-actin, anti-pAkt^{Ser473}, anti-Akt, anti-phospho-p44/p42 MAPK (pERK1/2) (Cell Signaling Technology, Denvers, MA); anti-EP4 (Cayman Chemicals, Ann Arbor, MI); Anti-COX-2 (BD Biosciences, Mississauga, ON, Canada); anti-ERK1/2 (Assay Designs, Inc. Ann Arbor, MI). After three washes (10 min each with TTBS), the membrane was incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 30 min at room temperature and again washed 3 times in TTBS for 10 min. The immunoreactive protein bands were visualized by Western Lighting® chemiluminescence reagent plus (Perkin Elmer, Boston, MA). To ensure equal protein loading, the same blot was stripped in Restore® western blot stripping buffer (Thermo-Scientific, Rockford, IL) for 45 min, washed in PBS and reprobed with appropriate antibodies for loading control.

HIF-1α Transcriptional Factor Assay

ED-273b and U-251 cells were seeded at a density of 3 x 10^6 cells onto culture dishes and were either untreated (normoxia), hypoxic treatment alone, or in the presence of 1μM PGE_2, and in the increasing concentrations of PGE_2 (2.5 - 20 μM) for 6 h. The nuclear extracts were prepared using NE-PER® Nuclear and Cytoplasmic Extraction Reagents kit as described above and frozen at -80°C as required. HIF-1α binding to the hypoxia response element was assessed using a commercial HIF-1α transcriptional factor assay kit (Cayman Chemicals, Ann

Arbor, MI) according to the manufacturer's protocol. In brief, a double stranded DNA sequence containing the HIF-1α response element (5'-ACGTG-3') is attached the wells of a 96-well plate. The prepared HIF-1α contained in nuclear extracts binds specifically to this DNA sequence. The HIF-1α transcription factor complex was detected by anti-HIF-1α antibody followed a secondary horseradish peroxidase-conjugated antibody. The color developed was monitored by taking absorbance measurements at 450 nm using a Benchmark microplate reader Model 680 (Bio-Rad Laboratories, Hercules, CA).

Human Angiogenesis Antibody Array

ED-273b cells seeded at a density of 3 x 10^6 cells onto culture dishes were exposed to either hypoxic treatment (1% O_2) in the presence or absence of PGE_2 or normoxic treatment at 21% O_2. After 6 h incubation, the culture medium was collected and subjected to screening for angiogenesis-related protein release using human angiogenesis antibody proteome profiler® kit (R & D systems, Minneapolis, MN) according to the manufacturer's instructions. In brief, the precoated antibody membrane was blocked in BSA blocking buffer and then incubated with the samples which were premixed with a cocktail of antibodies overnight and then followed by secondary horseradish peroxidase (HRP)-conjugated streptavidin antibody detection. The immunoreactive dot signals in duplicate on the membrane was detected by Western Lighting® chemiluminescence reagent plus (Perkin Elmer, Boston, MA) and visualized by

exposure on x-ray film for 5 to 10 min. The dot mean pixels density was quantified by Image J software and normalized to positive controls.

Statistical Analysis

Statistical analyses were performed utilizing a two-tailed, unpaired Student *t*-test using a significant threshold of $p < 0.05$.

4.3. Results

Exposure to Hypoxia Causes COX-2 Upregulation and Increase in PGE_2 Levels

Our previous studies (Chapters 2 and 3) demonstrated that primary glioblastoma ED-273b cells secrete relatively high amounts of COX-2-derived PGE_2 which promotes cell proliferation and invasion. Several studies have shown that PGE_2 plays a key role in hypoxia-induced VEGF expression in colon (42) and prostate (46) cancer cells. To investigate whether this process is also involved in glioblastoma cells, we first examined the effects of COX-2 expression and PGE_2 levels in response to hypoxia treatment. To minimize the effect of growth factors on COX-2 expression, primary glioblastoma ED-273b cells were serum-starved for 24 h and then exposed to 1% O_2 (hypoxia) for a period of time from 0 to 48 h. Immunoblot analysis revealed that hypoxia led to a detectable HIF-1α protein level within 3 h, and a maximal induction of HIF-1α was observed at 6 h and

thereafter returned to basal levels within 24 – 48 h (**Figure 4-1A**). Similarly, COX-2 expression was induced with peak levels observed within 6-16 h after exposure to hypoxia (**Figure 4-1A**). Notably, hypoxia caused a 2-fold increase in PGE_2 levels released into the spent media from cells at 6 h when compared with the normoxic (21% O_2) cells at 6 h. PGE_2 levels remained significantly elevated for 16 h and reduced to basal levels at 24 h when compared with the time points at normoxic conditions (**Figure 4-1B**). Of note, the increase in COX-2 expression in response to hypoxia was associated with a comparable increase in PGE_2 levels, suggesting an association of COX-2 expression and PGE_2 levels during hypoxia.

PGE_2 Potentiates Hypoxia-induced HIF-1α Protein Expression and Transcriptional Activity

To investigate a possible interplay between COX-2/PGE_2 and HIF-1α pathways, we next determined whether PGE_2 affects HIF-1α expression and its transcriptional activity. Since the GBM cells response to hypoxia maximized at 6 h, we chose this time window for subsequent experimental studies. Serum-starved ED-273b and U-251 glioblastoma cells were treated for 6 h at 1% O_2 with 1 μM PGE_2 or increasing concentrations of PGE_2, as indicated, and the nuclear extracts were isolated. Immunoblot analysis showed that under hypoxia, exogenously added PGE_2 (1μM) stimulation enhanced the effect of HIF-1α protein expression in both cell lines, leading to a greater accumulation of HIF-1α protein (**Figure 4-2A**). Furthermore, the effect of PGE_2 on HIF-1α expression was a "bell-shaped"

dose-response relationship in both PGE_2-treated ED-273b and U-251 cells, suggesting differential activation of EP receptors at different concentrations of PGE_2 (**Figure 4-2B**), which is in agreement with a previous report for the treatment of colorectal cancer (47). The corresponding samples used for western blotting were also assayed for HIF-1α transcriptional activity by ELISA assay. We showed that under hypoxia, PGE_2-induced HIF-1α accumulation correlated with a significantly increased activation of the transcriptional HIF-1α activity, compared with hypoxia treatment alone (**Figure 4-2C**). In addition, PGE_2 treatment alone stimulated hypoxia-induced HIF-1α transcriptional activity which was correlated with the PGE_2-induced HIF-1 expression at the similar concentrations (**Figure 4-2D**), indicating that the increased HIF-1α protein expression involves its transcriptional activity. These results confirm that, like hypoxia, PGE_2 signaling also enhances the HIF-1α transcriptional activity and the expression of HIF-1α protein.

PGE_2 Enhances HIF-1α Protein Synthesis

Under normoxic conditions, endogenous HIF-1α protein is rapidly degraded through the interaction of prolyl hydroxylase and the von Hippel-Lindau (VHL) protein resulting in a half-life of less than 5-min. Hypoxia causes a decreased rate of degradation of HIF-1α, thereby stabilising the protein. To determine whether PGE_2-induced HIF-1α expression involves a decreased rate of protein degradation or increased protein synthesis, we exposed cells for 4 h to $CoCl_2$ (HIF-1α protein

degradation inhibitor) which is similar to the effect of hypoxia, or 6 h in the hypoxia chamber in the presence or absence of 1 μM PGE_2. Thereafter, cycloheximide (CHX), a protein synthesis inhibitor, was added for 15-60 min. In cells exposed to hypoxic mimetic agent $CoCl_2$, the half-life of HIF-1α was > 60 min despite the lack of ongoing protein synthesis (**Figure 4-3A**), consistent with another study showing that $CoCl_2$ blocked HIF-1α protein degradation (42). Under hypoxic conditions, cells treated with PGE_2 followed by CHX led to a decrease in the half-life of HIF-1α protein < 30 min and HIF-1α was no longer detected after 60 min. (**Figure 4-3B**). These results suggest that PGE_2 signaling under hypoxic conditions enhances HIF-1α protein levels which are dependent on ongoing protein synthesis to increase its rate of protein expression, possibly through a translation-dependent pathway.

Hypoxia-induced Upregulation of COX-2 Enhances PGE_2 and VEGF Levels

Previous studies have shown that COX-2 is a hypoxia-responsive gene in colorectal tumor cells (39) and its metabolite PGE_2 plays key role in colon tumor progression (48). Having shown hypoxia-induced up-regulation of COX-2 expression in glioma cells, we were interested in determining whether the increased level of PGE_2 is indeed COX-2-dependent during hypoxia. In this study, cells were exposed to hypoxia for 6 h in the presence or absence of a physiological dose of 10 μM celecoxib, which has been shown to inhibit PGE_2 production by >80% and was not associated with down-regulation of COX-2

protein levels (49). As shown in **Figure 4-4A**, hypoxia causes a three-fold increase in PGE_2 release levels of the hypoxic ED-273b cells, compared with normoxic cells whereas this increase was not seen in the COX-2 deficient glioblastoma U-251 cells. In addition, the increased PGE_2 levels was significantly reduced in the hypoxic as well as normoxic cells pre-treated with low dose celecoxib (10μM), suggesting that the increased PGE_2 levels are most likely derived from the upregulation of COX-2 during hypoxia.

Although the underlying pathways responsible for GBM angiogenesis are to be fully explored, most studies have focused on the hypoxia-driven VEGF paradigm. Given that PGE_2 upregulates the transcriptional activity of HIF-1α under hypoxic conditions in GBMs, we investigated the modulating effect of PGE_2 on VEGF function in concert with the activity of HIF-1α. Western blot analysis showed that PGE_2 stimulation alone or combined with pre-treated celecoxib in ED-273b cells failed to induce HIF-1α expression under normoxic conditions whereas in hypoxia conditions, PGE_2 stimulation alone enhanced HIF-1α expression (**Figure 4-4B**). Interestingly, pre-treatment of cells with 10 μM celecoxib (CXB) for 24 h inhibited the basal hypoxia-induced HIF-1α accumulation; but subsequent addition of PGE_2 restored the inhibitory effect of celecoxib on HIF-1α expression (**Figure 4-4B**). Accordingly, PGE_2 treatment caused a 2.5-fold increase in VEGF release into the culture medium and subsequent exogenously added PGE_2 restored the inhibitory effect of CXB on the VEGF release (**Figure 4-4C**). A similar effect was also seen in HIF-1α transcriptional activity. Under hypoxia, PGE_2 potentiated HIF-1α activity and

rescued the inhibitory effect of CXB on HIF-1α transcriptional activity (**Figure 4-4D**). These findings clearly show that changes in HIF-1α protein expression correlate well with endogenous COX-2 activity. Thus, PGE_2 enhances hypoxia-induced VEGF production in glioblastoma cells, highlighting the critical role of the COX-2/PGE_2 pathway in modulation of VEGF levels under the hypoxic microenvironment.

PGE_2 Induces the Expression of Angiogenesis-related Proteins in Hypoxic Cells

To determine whether PGE_2 stimulation during hypoxia may also modulate the expression of several other HIF-1α or non-HIF-1α target genes, we performed an antibody array, which is a western blot-based immunoscreening assay that allows simultaneous assessment of a panel of 100 proteins of interest in a single assay. Cells untreated (normoxia) or treated with 1% O_2 (hypoxia) for 6 h in the presence or absence of 1 μM PGE_2 and thereafter subjected to the immunoscreening assay for the expression profile of 55-angiogenesis-related proteins in the spent culture medium. We observed PGE_2-induced upregulation of 8 of 55 proteins that are known to contribute the increased angiogenesis in cancer cells. Our results reveal that PGE_2 stimulation caused a significant 3.6-fold increase in interleukin 8 (IL-8); ~2-fold increases in monocyte chemotactic protein 1 (MCP-1), insulin-like growth factor binding protein 3 (IGFBP-3), pentraxin-3 and matrix metalloproteinase 9 (MMP-9) (**Figure 4-5**). As noted, PGE_2 triggered smaller increases of VEGF, granulocyte macrophage colony-

stimulating factor (GM-CSF) and tissue inhibitor of metalloproteinase 1 (TIMP-1), as compared to cells cultured in normoxic conditions (**Figure 4-5**). This gradient of growth factors and cytokines induced by PGE_2 in hypoxic cells has been previously shown to be factors that promote tumor cell growth, angiogenesis and invasion/metastasis. This variety of angiogenesis related factors operating within the GBM microenvironment is likely responsible for tumor cell redundancy and resistance to single anti-angiogenesis therapies. This assumption is supported by a recent study from Lucio-Eterovic *et al.* showing that blocking VEGF activity with bevacizumab (Avastin) in GBM was associated with upregulation of other angiogenesis factors (8).

The Effects of PGE_2-induced HIF-1α and VEGF Expression Involves EP4 Receptor Activity and Multiple Downstream Targets

Having shown the hypoxic effects of PGE_2-induced enhanced HIF-1α and VEGF expression, we aimed to gain insight into the cellular signaling pathways and mechanism(s) involved in HIF-1α/VEGF-induced angiogenesis. It is well recognized that PGE_2 exerts its biological activity via four G-protein-coupled receptor subtypes called EP1, EP2, EP3, and EP4. In Chapter 3, we showed that ED-273b cells utilized EP4 as the predominant receptor for growth-promoting effects of PGE_2. In this study, we demonstrate that under hypoxia, EP4 agonist PGE_1-OH treatment at low concentration was as effective as PGE_2 treatment in stimulating of HIF-1α expression in the nuclear extracts of ED-273b cells (**Figure**

4-6A). As expected, pharmacologic antagonist EP4 (ONO-AE3-208) significantly attenuated the expression levels of HIF-1α protein, whereas exogenously added PGE_2 restored the inhibitory effect of HIF-1α expression in both cell lines (**Figure 4-6B**). Similarly, EP4 antagonist ONO-AE3-208 suppressed the PGE_2-stimulated HIF-1α transcriptional activity by 2-fold in ED-273b cells, compared with the PGE_2 treatment alone (**Figure 4-6C**). From these studies we conclude that under hypoxia conditions, PGE_2 requires the EP4 receptor to enhance the HIF-1α protein expression in glioblastoma cells.

Several studies have shown that the increased HIF-1α stability and subsequent VEGF expression are dependent on signals mediated by ERK1/2 (50, 51), Src (52) and PI3K/AKT (53-55) signaling pathways. To further investigate the cellular signaling pathways involved in PGE_2 stimulation, we pre-treated ED-273b cells with various pharmacologic antagonists of MEK, PI3K and Src kinase activity and thereafter applied hypoxic treatment for 6 h in the presence or absence of 1 μM PGE_2. Western blot analysis demonstrates that PGE_2 stimulation caused an increase of HIF-1α protein expression and a relatively small increase of VEGF expression in hypoxic cells pre-treated with pharmacologic antagonists of MEK (PD98059) or PI3 kinase (LY294002), as compared with non-treated PGE_2 hypoxic cells (**Figure 4-6D**). Interestingly, this increase was also accompanied with the increased activation of ERK1/2 (the increase in phosphorylation of ERK1/2). In contrast, PGE_2 treatment had no predominant effects on hypoxia-induced HIF-1α and VEGF expression as well as ERK1/2 activity (phosphorylation of ERK1/2) in hypoxic cells pre-treated with pharmacologic

antagonist of Src (PP2), compared with non-treated PGE_2 hypoxic cells (**Figure 4-6D**). These results indicate that there exists a novel compensatory pathway influenced by either the ERK1/2 or PI3K/Akt pathways.

4.4. Discussion

Considerable evidence supported by clinical and *in vitro* studies has demonstrated that hypoxia not only induces tumor angiogenesis but also increases tumor invasion and metastasis (56-58). In most other cancers, patient death is attributable to metastasis and not the primary tumor growth (59). GBM, a highly hypoxic and diffusely infiltrative tumor, depends on angiogenesis for the proliferation and survival through hypoxia driven up-regulation of VEGF activity (58). Despite recent advances in anti-VEGF targeted therapy, most patients with GBM demonstrate a short-lived response. One of the possible mechanisms attributable for resistance to anti-angiogenic therapy may result from glioblastoma cells adapting by migrating more aggressively into normal brain tissue via co-option normal blood vessels (60, 61) or up-regulation of alternative angiogenic signaling circuits (e.g. angiogenin, IL-8, FGF-2). Thus, an expanded understanding of hypoxia-induced VEGF and other angiogenesis signaling pathways (i.e. IL-8, angiogenin, FGF-2, etc) will be required to identify more efficacious strategies that will overcome the angiogenesis redundancy observed in GBM.

In this study, we have depicted some of the molecular pathways by which PGE_2 stimulates VEGF production in a mimetic hypoxia environment, thereby providing a cross-talk association between COX-2 and HIF-1α activity in promoting tumor angiogenesis. We demonstrate that hypoxia triggers an increased release of PGE_2 via induction of COX-2 expression and that PGE_2 enhances HIF-1α protein expression and transcriptional activity under hypoxic conditions mediated through the EP4 receptor. In addition, we show that PGE_2 modulates the levels of VEGF in concert with COX-2 activity.

Overexpression of COX-2 has been reported in primary and recurrent gliomas (62-65). The levels of COX-2 expression have correlated with increased tumor grade and are associated with decreased overall patient survival (66, 67). Inhibition of COX-2 activity putatively improves glioma clinical outcomes (65). However, the regulatory mechanisms that underlie glioma COX-2 up-regulation have largely remained unexplored. Our study demonstrates for the first time that COX-2 is induced by hypoxia in glioblastoma cells and that this induction is mediated in part by HIF-1α. These findings correlate with other studies showing that COX-2 is up-regulated via the HIF-1α pathway in hypoxic colon (39) and lung (68) cancer cells. In addition, COX-2 upregulation by hypoxia has been shown previously in human corneal endothelial (38) and umbilical vascular endothelial cells (40, 69) and is mediated through peroxisome proliferator-activated receptors and NF-κB, respectively. Although we did not provide direct evidence to show that HIF-1α directly binds to HRE on the COX-2 promoter as reported by others (39, 68), it is assumed that the COX-2 promoter in ED-273b

glioblastoma cells contains a functional core sequence of the HRE that binds HIF-1α directly in glioblastoma. Furthermore, we showed that inhibition of COX-2 activity by a selective COX-2 inhibitor celecoxib at a physiological dose of 10 μM, shown to have no effect of cell growth and induction of apoptosis (49, 70) resulted in decreased basal levels of HIF-1α and its transcriptional activity. We also showed that exogenously added PGE_2 to celecoxib-treated cells restored the ability of hypoxia to induce HIF-1α expression and activity, suggesting that COX-2 derived PGE_2 is involved in the biological activity of the hypoxic microenvironment of GBMs.

GBM is a highly vascular and hypoxic tumor characterized by "pseudopalisading" necrosis and microvascular hyperplasia. It has been suggested that the intra-tumor pseudopalisades represent a wave of hypoxic tumor cells migrating away from the central hypoxia anoxic regions (31, 71). However, the underlying mechanism(s) of pseudopalisading cell formation in GBM remain elusive. Here, we show that hypoxia treatment results in the enhanced PGE_2 production via upregulation of COX-2 expression in ED-273b cells. Given the growth-promoting effect of PGE_2 in GBM as shown in Chapter 3, this finding can explain, at least in part (along with glycolytic metabolism) how glioblastoma cells maintain their growth and survival under the hypoxic tumor microenvironment (12, 72). Our data indicate that elevated levels of PGE_2-induced VEGF plays a pivotal role in the provision of adaptive cellular responses which allows hypoxic solid tumor such as glioblastoma to thrive during tumor initiation and tumor expansion.

Several studies have suggested that inhibition of COX-2 activity causes a decrease in VEGF levels in prostate (73) and lung cancer cells (74), leading to impaired tumor growth and reduced angiogenesis. On the other hand, PGE_2 has been shown to promote the expression of VEGF mediated by HIF-1α in colon cancer cells (39, 42), supporting colorectal cell survival and angiogenesis. In line with these findings, we demonstrate that a physiological dose of celecoxib prevents hypoxia-induced up-regulation of VEGF and HIF-1α activity, while subsequent addition of PGE_2 reverses the inhibitory effect of celecoxib in glioblastoma cells. We propose that the increased VEGF expression induced by PGE_2 in glioblastoma ED-273b cells is an indirect angiogenic effect via the HIF-1α transcriptional activity for two reasons. First, it is known that the *VEGF* gene contains a HRE within its promoter which binds HIF-1α (75, 76). Second, we provide evidence that PGE_2 potentiates the HIF-1α transcriptional activity which may further enhance the induction of VEGF to promote angiogenesis. Taken together, these findings indicate the presence of a novel autocrine feedback loop that exists in ED-273b COX-2 expressing cells. Hypoxia induces the expression of COX-2 which causes the increase of PGE_2 levels and thereby in return, amplifying the HIF-1α transcriptional activity to enhance VEGF production in the hypoxic GBM microenvironment.

Previous studies have shown that HIF-1α is overexpressed in human primary and metastatic tumors (77-80). In GBM, up-regulation of HIF-1α can be detected in pseudopalisading cells surrounding necrotic regions and in tumor cells infiltrating normal brain tissues (30, 81). In addition, increased HIF-1α activity is

correlated with increased glioma tumor grade and cell invasiveness, VEGF induction and stimulation of angiogenesis (11, 30). HIF-1α can also be regulated by cytokines (82, 83) and PGE_2 (42). Although the PGE_2-mediated hypoxic effect on VEGF production in prostate cancer PC-3ML cells were attributed to stabilization of HIF-1α protein (44), we demonstrate that even with prolonged hypoxia, HIF-1α protein was nearly completely degraded within 30 min in PGE_2-treated cells following inhibition of protein synthesis. In agreement with previous studied (39, 42), our data suggest that under hypoxic conditions, PGE_2 up-regulates HIF-1α by increasing the rate of protein synthesis, which subsequently enhances VEGF secretion.

Several studies have indicated that the ERK1/2 and PI3-K signaling pathways modulate hypoxia-induced effects of HIF-1α expression (53, 84, 85), with the former mediating HIF-1α transcriptional activity (50) and the latter mediating HIF-1α rate of protein synthesis (54). In addition, another study revealed that PGE_2 activates the ERK1/2 pathway via EP2 and EP4 receptors in prostate cancer cells (44) and the ERK1/2 and PI3K pathways via the EP1 receptor in colon cancer cells (42). Our study shows that PGE_2 exerts its action via EP4 receptor, mediating the hypoxia-induced effect of HIF-1α expression and that inhibition of EP4 receptor by the pharmacologic antagonist ONO-AE3-208 attenuates PGE_2-induced HIF-1α protein production. To our knowledge this is the first study to show that PGE_2 signaling requires the EP4 receptor to stimulate hypoxia-induced HIF-1α up-regulation in primary glioblastoma cells *in vitro*. In addition, our results also demonstrate that pretreatment with LY294002 or

PD98059 in the presence of PGE_2 appears to upregulate the PGE_2-induced HIF-1α and VEGF expression. It is likely that increased activity of the ERK1/2 pathway (shown by the increased phosphorylation of ERK1/2) generated through inhibition of either the ERK1/2 or PI3K pathways by the pharmacologic antagonists contributes to the dramatic increase expression of HIF-1α and thereafter VEGF induction via a compensatory pathway in ED-273b glioblastoma cells. This is not surprising, as the activation of ERK1/2 is sufficient to promote the transcriptional activity of HIF-1α and HIF-1α expression which in turn upregulates VEGF induction (50). More recently, the activity of ERK1/2 pathway has been shown to be a key signaling pathway exposed to hypoxia and the development of an invasive phenotype in GBM cells (86). Interestingly, inhibition of Src kinase activity fails to block the hypoxia-induced basal HIF-1α expression and marginally reduces the PGE_2-induced HIF-1α and VEGF expression, suggesting that the Src pathway is not associated with the hypoxia-induced HIF-1α and VEGF induction. Our data indicate that the PGE_2 and EP receptor-dependent signals that promote HIF-1α stabilization or protein synthesis under hypoxic conditions are complex signaling networks which involve multiple pathways. Further studies are required to delineate these complex pathways and the specific roles of PI3K, ERK1/2 and Src in PGE_2-induced HIF-1α protein expression as well as VEGF induction in glioblastoma cells.

In addition to the HIF-1α-dependent pathway, hypoxic induction of VEGF expression can also be regulated via several HIF-1α-independent pathways such as basic fibroblast growth factor (87), dysregulated ERK1/2 and PI3K signaling

pathways (37), transcriptional factor NF-κB (88) or oncogenic K-Ras (89). Our data demonstrate that PGE_2 stimulation causes a 3.6-fold increase in IL-8 and ~2-fold increase in MCP-1, IGFBP-3, pentraxin-3 and MMP-9 in hypoxic cells. Among the gene targets of HIF-1α, MCP-1 (90) and IGFBP-3 (91) are relevant to cellular invasive behaviour and have been shown to be involved in the induction of HIF-1α-mediated VEGF (92). In contrast, both IL-8 and MMP-9 are known to be the gene targets of NF-κB and are also involved in glioma invasion and angiogenesis (93-95). Of note, the proangiogenic cytokine IL-8 can act as a compensatory pathway in the absence of HIF-1α via the NF-κB pathway to preserve the angiogenic response in colon cancer cells (94). This cytokine has also been shown to have direct growth-stimulating, migrating and angiogenic effects in gliomas (96, 97). Notably, only a small increase of the VEGF induction in response to PGE_2 stimulation was detected by the angiogenesis antibody array when compared with the ELISA assay which yielded a 2.5 fold increase in VEGF induction (**Figure 4-3C**). This result could have been caused by "oversaturation" of the VEGF antibody on the membrane of the array kit. Further dilution of the assay samples in future studies will help to resolve this problem. Taken together, our results suggest that PGE_2-induced IL-8 secretion during hypoxic conditions could be a long-term adaptive response utilized by GBM cells as an additive or alternative pathway for glioma angiogenesis independent of HIF-1α. Further investigation is required to validate these findings.

In summary, our results show that under hypoxic conditions, PGE_2 signaling via the EP4 receptor activates the HIF-1α transcription and protein

expression through multiple signaling pathways and promotes enhanced VEGF induction. The data presented in this study suggest that increased HIF-1α activity synergized through COX-2/PGE_2 signaling may contribute significantly to the initiation of the angiogenic switch during tumor transformation and progression. Our studies implicate that compounds that simultaneously inhibit multiple cross-talking signaling pathways may represent a viable focus of new anti-angiogenic and anti-invasive therapies for GBMs. Drugs targeting the EP4 receptor may complement anti-HIF-1α, selective COX-2 and/or PGE_2 synthase inhibitors, in abrogating angiogenesis and proliferation, leading to the eradication of these aggressive glioblastoma tumors.

Figure 4-1. Effect of hypoxic treatment on COX-2 expression and PGE_2 secretion. Serum-starved primary culture of glioblastoma ED-273b cells were incubated under normoxic (21% O_2) and hypoxic (1% O_2) conditions for 3, 6, 16, 24, and 48 h. **A**, cells were lysed and proteins in the nuclear extracts were isolated. The cell lysates (50 μg) were immunblotted with anti-HIF-1α and anti-COX-2 antibodies and the same blot was stripped and reprobed with anti-β-actin antibody for total protein loading control. **B**, the spent culture medium of normoxic and hypoxic treated cells was collected and subjected to PGE_2 measurement by EIA according to the manufacturer's protocol. Results shown were plotted as average mean ± SEM in duplicate of three independent experiments standardized to control untreated cells. (*, $p<0.05$ vs. normoxia at each time points)

Figure 4-1.

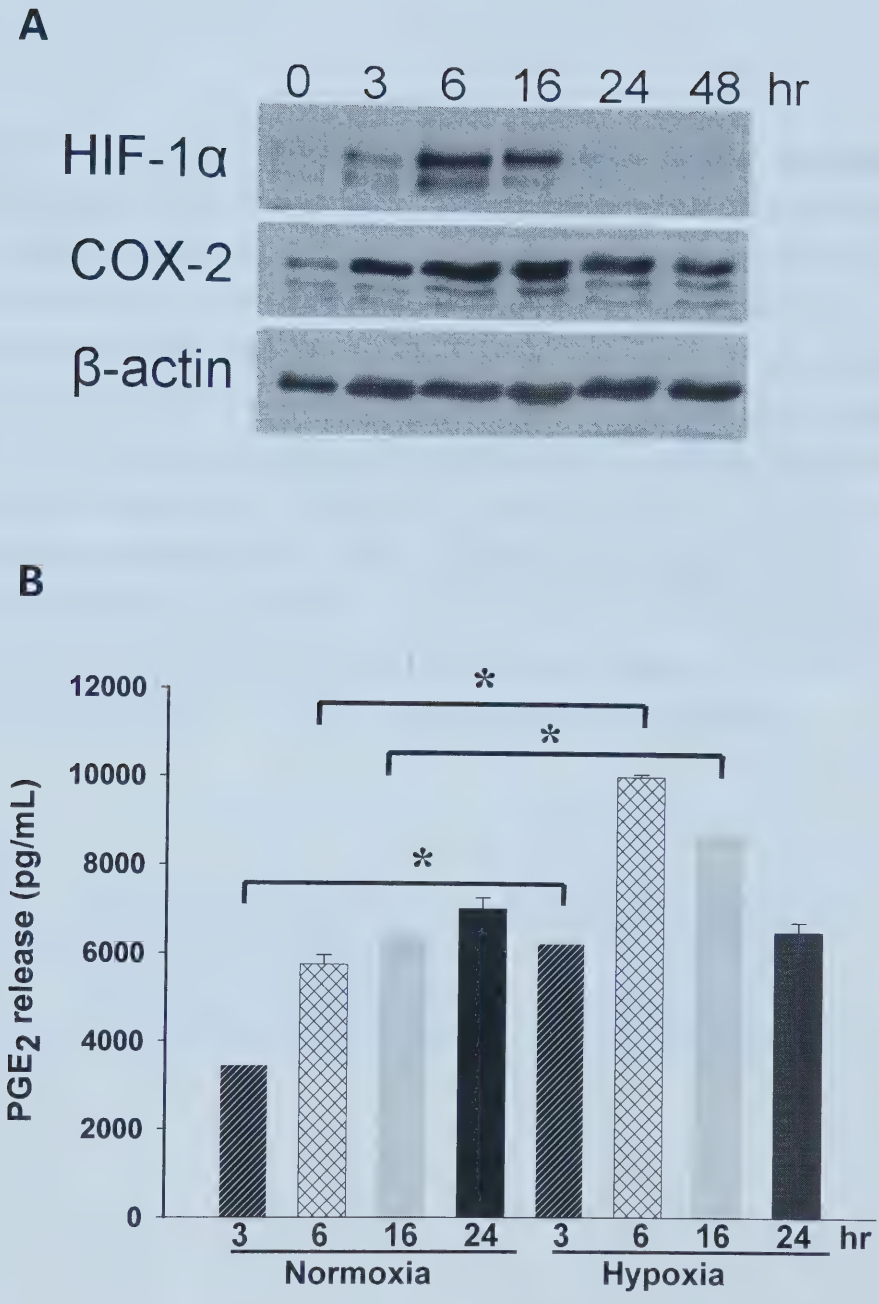

A
0 3 6 16 24 48 hr
HIF-1α
COX-2
β-actin
B
PGE2 release (pg/mL)
12000
10000
8000
6000
4000
2000
0
*
*
*
3 6 16 24 3 6 16 24 hr
Normoxia
Hypoxia

Figure 4-2. Effect of PGE_2 treatment on HIF-1α protein expression, transcriptional activity and stability. Primary culture of ED-273b and long-term established culture of U-251 glioblastoma cells were untreated (normoxia) or treated with PGE_2 at 1 μM concentration or increasing concentrations (2.5, 5, 10, 20 μM) under hypoxic conditions for 6 h. **A** and **B**, cells were lysed and proteins in the nuclear fractions were isolated. The cell lysates (30 μg) were immunblotted with anti-HIF-1α antibody and the same blots were stripped and reprobed with anti-β-actin antibody for total protein loading controls. **C** and **D**, the corresponding samples of the nuclear fractions were subjected to HIF-1α transcriptional activity measurement using a commercial HIF-1α transcriptional activity ELISA assay kit according to the manufacturer's protocol. (*, $p<0.05$ vs. normoxia or control(0), **, $p<0.05$ vs. untreated control (0) or hypoxia).

Figure 4-2.

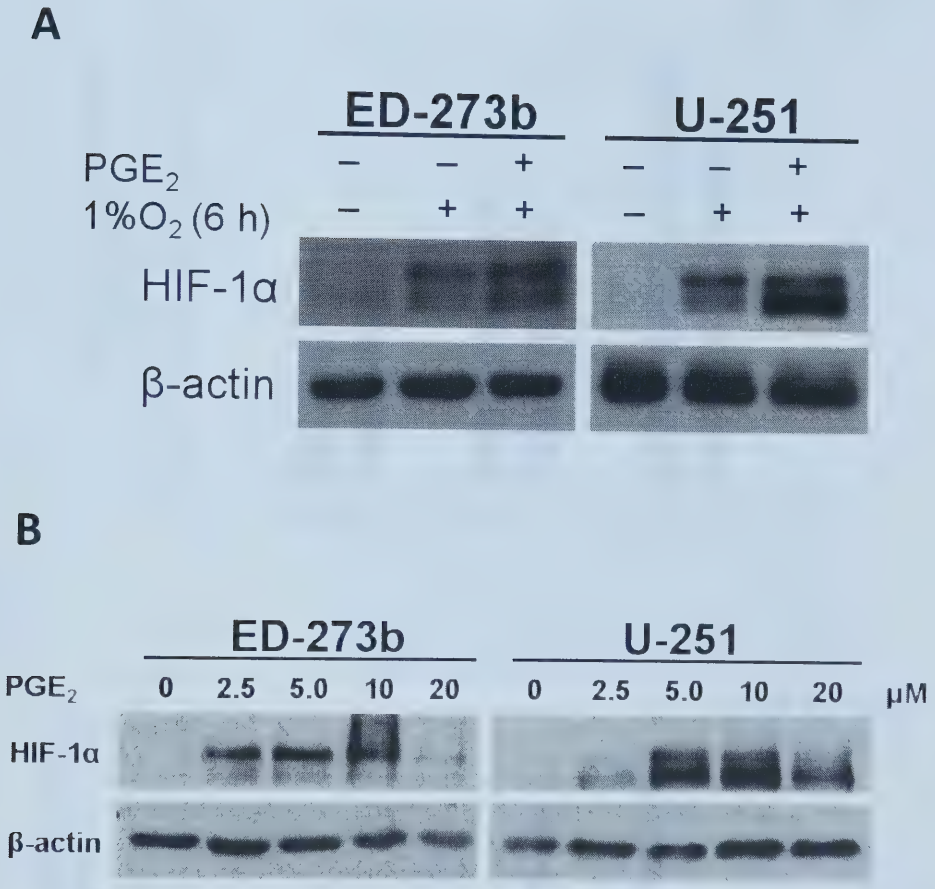
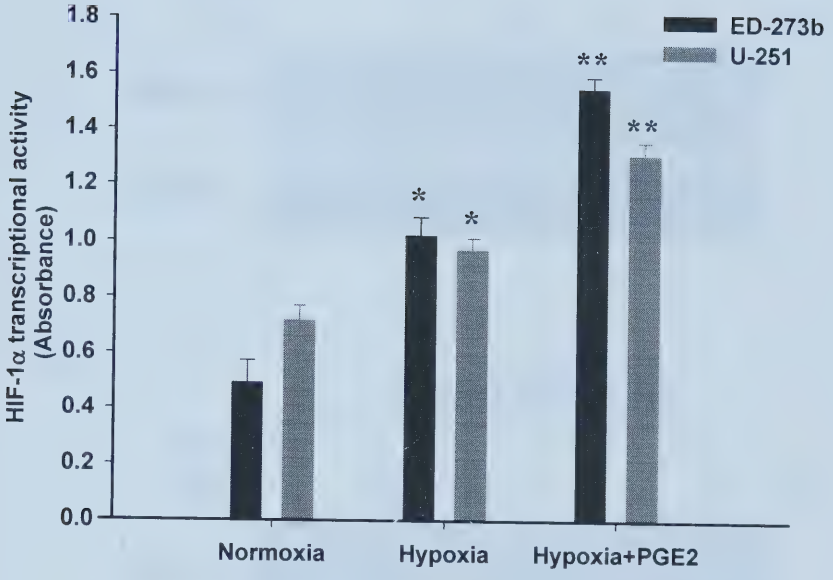

A
ED-273b
U-251
PGE2
1%O2 (6 h)
HIF-1α
β-actin
B
ED-273b
U-251
PGE2
0 2.5 5.0 10 20 0 2.5 5.0 10 20 μM
HIF-1α
β-actin

Figure 4-2

C


HIF-1α transcriptional activity
(Absorbance)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ED-273b
U-251
*
*
**
**
Normoxia
Hypoxia
Hypoxia+PGE2

D

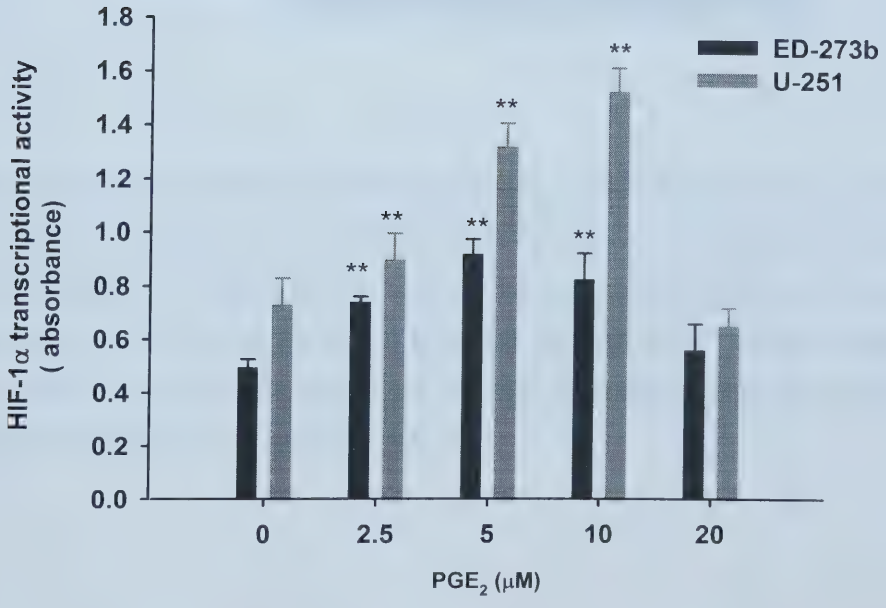

HIF-1α transcriptional activity
(absorbance)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ED-273b
U-251
**
**
**
**
**
0
2.5
5
10
20
PGE2 (μM)

Figure 4-3

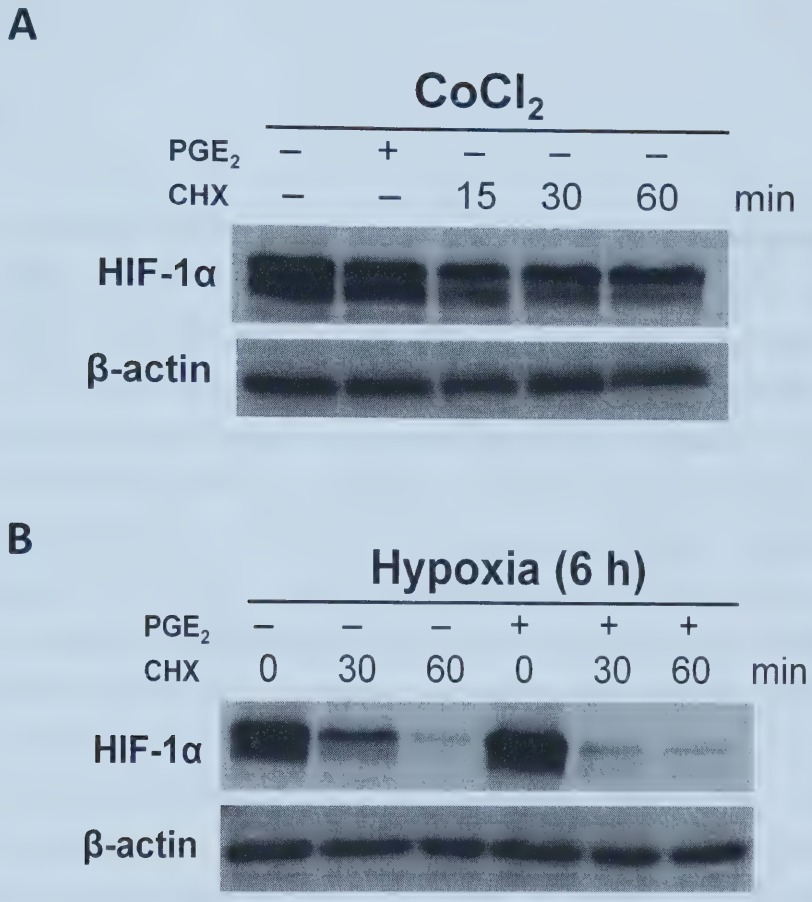


Figure 4-3. Effect of PGE_2 on HIF-1α turnover. ED-273b cells were exposed to 100 μM $CoCl_2$ (**A**) or hypoxia (**B**) for 6 h in the presence or absence of 1 μM PGE_2, cycloheximide (CHX) was added to a final concentration of 100 μM for an additional of 0, 15, 30, and 60 min, as indicated. Proteins in the nuclear extracts were isolated and immunoblotted with anti-HIF-1α antibody and anti-β-actin antibody for total protein loading controls.

Figure 4-4. COX-2 mediated hypoxic effects on HIF-1α transcriptional activity, PGE_2 and VEGF production. A, ED-273b and U-251 cells were pretreated with 10 μM celecoxib for 24 h followed by untreated (normoxia) or treated at 1% O_2 (hypoxia) for 6 h in the absence (control) or presence of celecoxib. The spent culture medium of normoxic and hypoxic treated cells were collected and subjected to PGE_2 determination. **B**, ED-273b cells were pretreated with 10 μM celecoxib for 24 h followed by 6 h untreated (normoxia) or treated at 1% (hypoxia) in 1 μM PGE_2 alone or in combination of celecoxib. The cell lysates were immunoblotted with anti-HIF-1α antibody and anti-β-actin antibody (protein loading control). The corresponding samples previously used for western blotting were also assayed for **C**, VEGF release in the culture medium and **D**, HIF-1α transcriptional activity by ELISA, as indicated. Results shown were plotted as average mean ± SEM in duplicate of two independent experiments standardized to control untreated cells. (*, †, $p < 0.05$ vs. control; **, ††, $p < 0.05$ vs. CXB).

Figure 4-4.

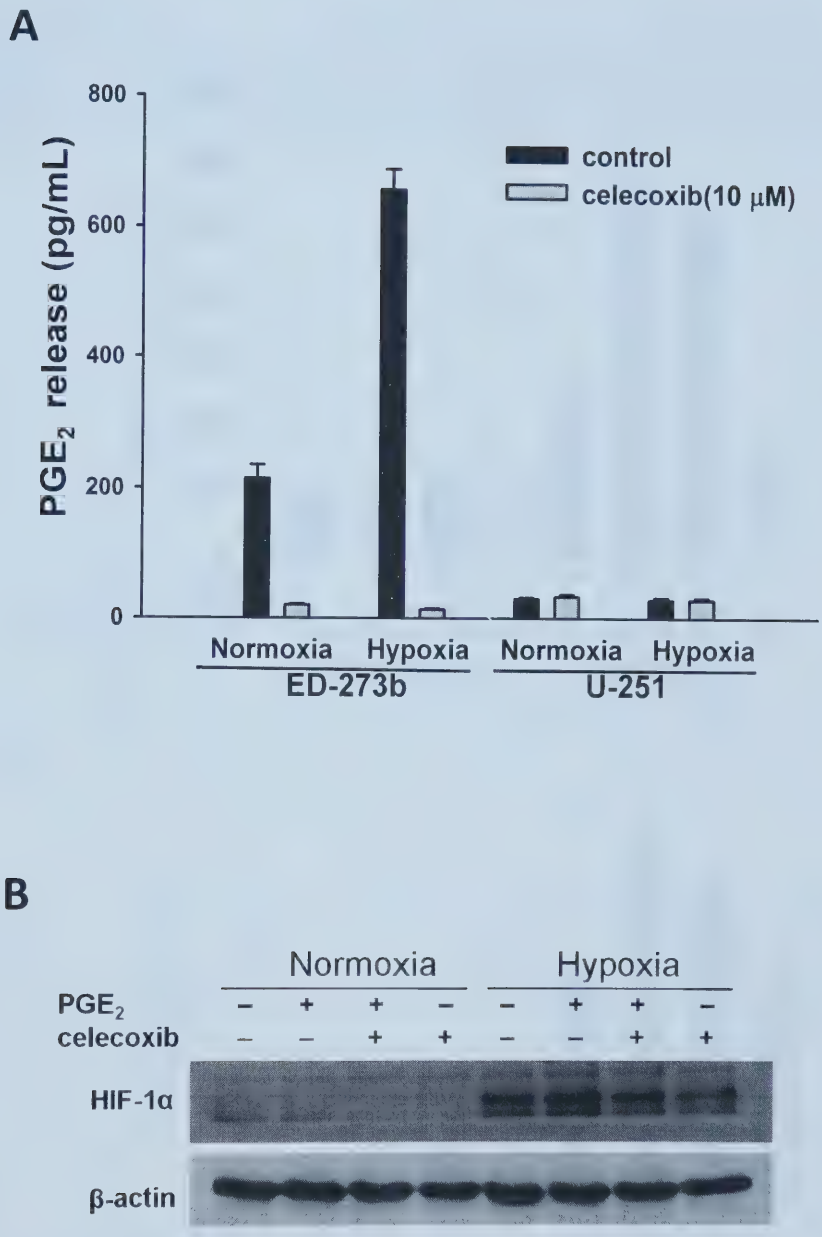

A
800
600
400
200
0
PGE2 release (pg/mL)
control
celecoxib(10 μM)
Normoxia
Hypoxia
Normoxia
Hypoxia
ED-273b
U-251
B
Normoxia
Hypoxia
PGE2
celecoxib
HIF-1α
β-actin

Figure 4-4.

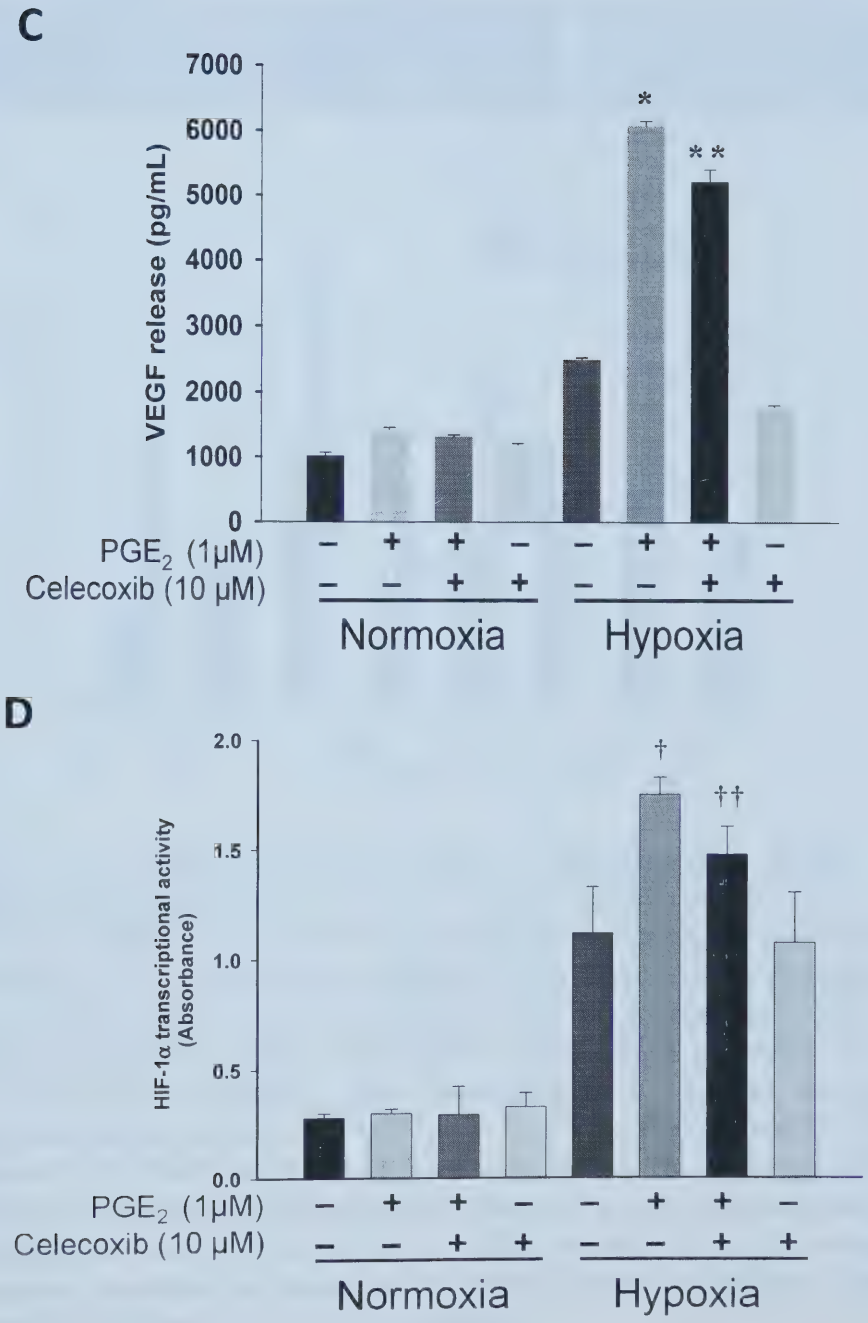

C
VEGF release (pg/mL)
7000
6000
5000
4000
3000
2000
1000
0
*
**
PGE2 (1μM)
Celecoxib (10 μM)
Normoxia
Hypoxia
D
HIF-1α transcriptional activity (Absorbance)
2.0
1.5
1.0
0.5
0.0
†
††
PGE2 (1μM)
Celecoxib (10 μM)
Normoxia
Hypoxia

Figure 4-5.

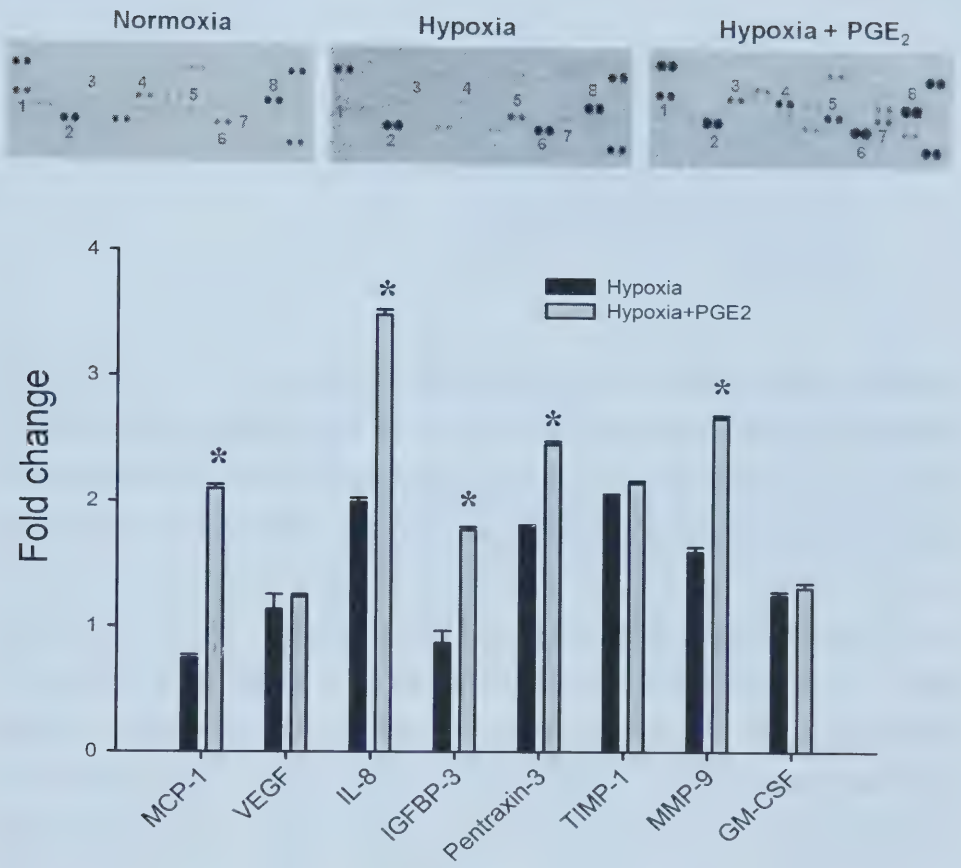


Figure 4-5. Hypoxic effects of PGE_2 on the expression profile of angiogenesis-related proteins. ED-273b cells were seeded a density of 3 x 10^6 in cultures dishes overnight. After serum-starved for 24 h, the cells were incubated at 21% O_2 (normoxia) or exposed to 1% O_2 (hypoxia) in the presence or absence of PGE_2 for 6 h. Each spent culture medium was collected and subjected to human angiogenesis antibody array analysis. Array images are shown on the top and the pair of duplicate spots representing by the number for each angiogenesis-related protein are MCP-1(1); VEGF(2); IL-8(3); IGFBP-3(4); pentraxin-3 (5); TIMP-1(6); MMP-9(7) and GM-CSF(8). The mean spot pixel densities are quantified by image-J software. Bars shown were mean fold change in angiogenesis-related protein expression ± SEM, as compared to cells cultured in normoxic conditions and normalized to positive controls in duplicate of two independent experiments. (*, $p < 0.05$ vs. hypoxia).

Figure 4-6. The impact of EP4 receptor and MAPK kinase mediated-effects of PGE_2-induced HIF-1α. **A**, ED-273b cells were treated with increasing concentrations of the EP4 agonist PGE_1-OH for 6 h, as indicated. N (normoxia), H (hypoxia). Western blot analysis was carried out to evaluate HIF-1α protein levels with β-actin as a loading control. **B**, ED-273b and U-251 cells were pretreated with EP4 antagonist 1 μM ONO-AE3-208 for 1 h followed by 1 μM PGE_2 for 6 h, or treated with the ONO-AE3-208, PGE_2 alone for 6 h under hypoxic conditions and analyzed by western blotting. **C**, the corresponding western blot samples of ED-273b cells described above were assayed for HIF-1α transcriptional activity. **D**, ED-273b cells were pretreated with 25 μM PD98059, 30 μM LY294002 and 10 μM PP2 for 1 h following by 1 μM PGE_2 in the presence of these inhibitors for 6 h under hypoxic conditions. Western blot analysis was used to assess HIF-1α and VEGF protein expression and ERK1/2 activation using anti-pERK1/2 antibody. ERK1/2 and β-actin were used as protein loading controls.

Figure 4-6.

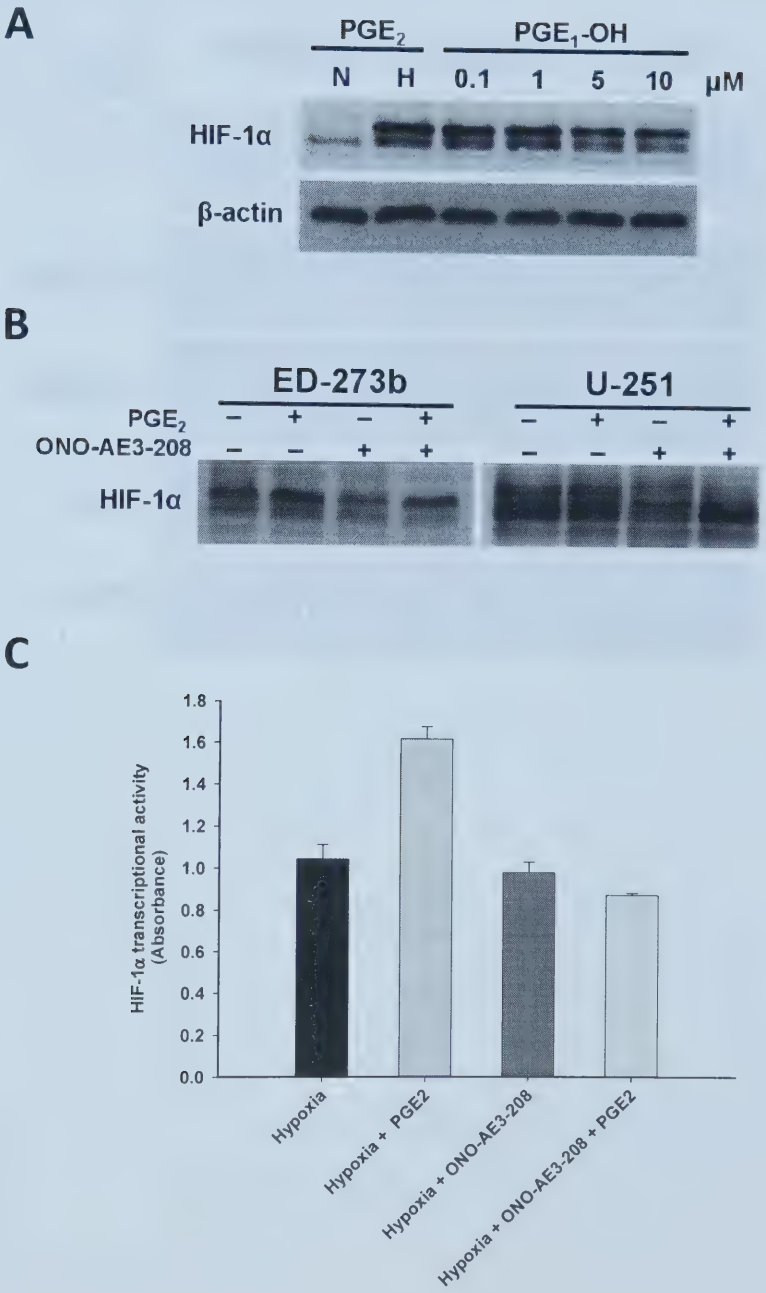
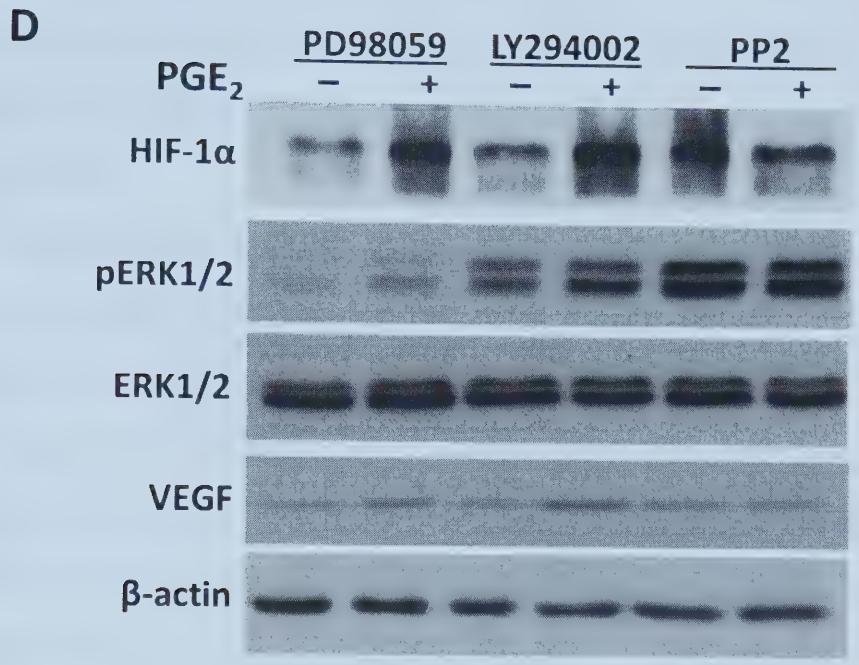

A
PGE2
PGE1-OH
N
H
0.1
1
5
10
μM
HIF-1α
β-actin
B
ED-273b
U-251
PGE2
ONO-AE3-208
HIF-1α
C
HIF-1α transcriptional activity (Absorbance)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Hypoxia
Hypoxia + PGE2
Hypoxia + ONO-AE3-208
Hypoxia + ONO-AE3-208 + PGE2

Figure 4-6.



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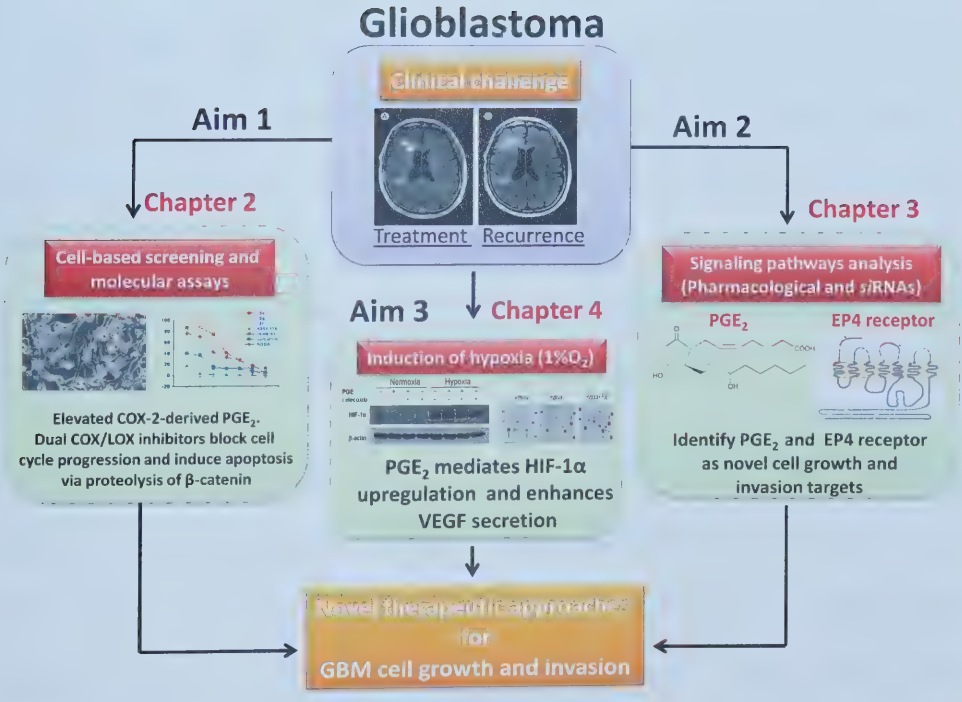
Chapter 5

Summary, Discussion and Future Directions



5.1. Summary

This thesis outlines several important aspects of human glioma biology including the molecular cross-talk between COX-2-PGE_2 and EGFR signaling in modulating glioblastoma tumor cell proliferation, growth and invasion. First, we review the experimental insights into COX-2/PGE_2 signaling in cancers and recent advances in glioma treatment and propose novel therapeutic strategies for the management of malignant gliomas. In the first study described in Chapter 2, we provide proof of principle that the selective putative dual COX/LOX inhibitors (compounds 13e, 13g and 13i) display a lack of appreciable biological COX-2 and 5-LOX inhibitory activity as reflected by cell-based screening assays. However, we demonstrate these compounds inducing both a G_0/G_1 phase block and apoptosis mediated through a cell-cell adherent pathway via a caspase-3 mediated proteolysis of β-catenin. Our findings suggest that these diarylprop-2-yn-1-one moiety compounds (1) could be considered as a lead compound for the development of a novel class of COX/LOX-independent chemotherapeutic agents for the management of cancers including high grade gliomas.

Our initial screening study of primary cultures (obtained from surgical specimens) and long-term established cultures show that human glioblastoma cells secrete elevated levels of PGE_2 which prompts us to the undertake of a number of experiments that investigate the prevalent receptor target of PGE_2 and downstream signaling function in relation to modulation of glioblastoma

tumorigenesis. As outlined in Chapter 3, we have identified a novel COX-2-derived PGE_2 signaling pathway which enhances glioblastoma cell proliferation and invasion through an EP4/Src-mediated transactivation of the EGFR, thereafter leading to the activation of both ERK1/2 and Akt mitogenic pathways. We further demonstrate that molecular and pharmacological inhibition of the EP4 receptor signaling was effective in suppressing the PGE_2-induced proliferation and invasion *in vitro*. This study emphasizes the molecular cross-talk between the COX-2-derived PGE_2 and EGFR signaling which is important in maintaining COX-2 overexpression and may provide access to novel combination therapies for high grade glioma tumors. In Chapter 4, we provide evidence that under hypoxic conditions, PGE_2 modulates HIF-1α transcriptional activity and VEGF secretion through the interplay between EP4/HIF-1α proteins, pro-angiogenic cytokines and its downstream effectors (i.e. ERK1/2 and Akt). These results support a critical role for PGE_2 in the angiogenic drive during glioma initiation and progression.

In summary, this thesis work reflects our efforts to elucidate a novel PGE_2 signaling pathway, orchestrating a cellular network of EP4 expression, EGFR signaling, and activation of Src, ERK1/2 and Akt kinases required for glioma cell proliferation and invasion. Future PGE_2 studies may be exploited through molecular and pharmacological methods to provide further rationale for utilizing EP4 receptor antagonist to synergistically compliment the use of selective COX-2 inhibitors to augment current anti-glioma therapies. Although these preliminary results are encouraging, expansion of knowledge in understanding the molecular

mechanisms underlying the PGE_2-EP4/Src signaling in glioma cell proliferation and invasion requires further in depth investigation. Many important questions remain to be explored and clarified by future studies.

5.2. Discussion and Future Directions

Although COX-2 and 5-LOX are promising therapeutic targets, how they regulate tumor growth and inhibit apoptosis continues to be explored and debated. It has been shown that inhibition of either COX-2 or 5-LOX leads to a "compensatory pathway" toward the unaffected active pathway (2, 3). Simultaneous inhibition of both arms of these pathways using combined COX-2/5-LOX inhibitors has additive effects in inhibiting cell growth and inducing apoptosis in various types of cancers (2-6). Thus, the search for new dual COX/LOX inhibitor compounds using structural-activity relationship (SAR) molecular modeling remains the focus of intensive investigations. Using this approach, we developed a series of novel 1,3-diarylprop-2-yn-1-ones moiety compounds including 13e, 13i and 13g (i.e. 13-series compounds) and showed encouraging anti-COX-2 and 5-LOX activity *in vitro* and *in vivo* (1).

As outlined in Chapter 2, we evaluated the 13-series compounds in a secondary cell-based screening approach for their biological effects of COX-2 and 5-LOX activities in glioblastoma cells. Although we found these compounds lacked an appreciable anti-COX-1/COX-2 and 5-LOX activities and suppressed

cell proliferation by inducing cell cycle arrest and apoptosis. This finding is in agreement with a report studying SAR analysis of diarylpyrazole pharmacophore modification for the potential use of dual COX-2/5-LOX inhibitors to suppress prostate tumor cell growth and induce apoptosis (7). We also provide evidence that the induction of apoptosis is mediated through cell-cell adherent pathway via a consequence of caspase-3 activation, leading to cleavages of β-catenin in glioblastoma cells as well as colon and prostate cancer cells. Furthermore, our results are supported by other studies which demonstrate the apoptotic cleavage of β-catenin with chemotherapeutic agent CDDP and paclitaxel (8, 9). However, several key issues remain to be explored. First, it will be necessary to establish whether these compounds are effective for the treatment of human GBM xenograft *in vivo* mouse models and the degree of potential off-target effects due to the requirement of high concentrations (40 to 60 μM) of the compounds to generate an apoptotic effect. This result is similar to celecoxib which also induces caspases-3 and caspase-9 mediated apoptosis but only at concentrations between 80 to 100 μM in colon cancer cells (10). Finally, more work is required to investigate the mechanisms of antitumor effect associated with β-catenin/TCF transcriptional activity using a TCF (T-cell factor) luciferase reporter gene (TOPFLASH; FOPFLASH) system. Nevertheless, our findings suggest that these putative dual COX-2/5-LOX inhibitors could be considered as a lead compound for the generation and optimization of a novel class of apoptosis-inducing chemotherapeutic agents which may be used as a monotherapy or in conjunction

with other chemotherapeutic agents in patients harboring these devastating tumors.

Although the biological effects of PGE_2 (proliferation, angiogenesis and invasion) and its prostanoid receptor function have been well characterized in colon cancers and in genetic knockout animal models (11-13), the role of PGE_2 in modulation of glioblastoma cell growth, angiogenesis and invasion has not been previously established. Our investigations described in Chapter 3 have revealed that COX-2-derived PGE_2 promotes cell proliferation via EP4/Src mediated transactivation of the EGFR, ultimately leading to activation of the PI3K/Akt and ERK1/2-MAPK mitogenic signaling pathways. However, the precise PGE_2 signaling cascade in glioma remains a salient feature of this investigation. It is important to know that the downstream signaling including components of MAPK and PI3K pathways are widely shared not only by GPCRs but also EGFR. In high grade gliomas, EGFR is commonly overexpressed and amplified (14); and EGFR signaling is one of the most important drivers of glioma proliferation and invasion. Several ligands bind to EGFR including transforming growth factor α (TGF-α) and heparin binding EGF (HB-EGF). Both HB-EGF and TGF-α participate in autocrine loops via EGFR signaling in glioma (15). Although our results show that PGE_2 stimulation triggers the kinase activities of ERK1/2 and Akt, the precise mechanism(s) by which PGE_2 activates MAPK and PI3K pathways are not fully determined and must be studied further. In this context, we cannot rule out the possibility that PGE_2 may indirectly increase the release of TGF-α or HB-EGF upon EGFR-stimulation via an autocrine loop to promote cell

proliferation. In future studies, we will measure the amounts of TGF-α and HB-EGF released into the culture medium upon PGE_2 treatment to resolve this issue. Our study shows that PGE_2 activates Src which enhances EGFR-mediated signaling to promote glioblastoma invasion and progression. Recently a similar cascade was found in an *in vivo* study in which a molecular cross-talk linking EGFR signalling and Src activation promoted glioblastoma invasion and survival (16). In addition, several other upstream targets such as integrin-linked kinase (ILK) (17) and PTEN (18) also regulate the activity of PI3K whereas Ras was shown to modulate activity of the ERK1/2-MAPK pathway (19). Finally, it has been shown that COX-2-derived PGE_2 mediates the activation of RAS-MAPK pathway which in turn upregulates COX-2 expression to promote adenoma cell growth by autoregulation of COX-2 expression (19). Therefore, our studies support a novel positive feedback mechanism by which COX-2-derived PGE_2 promotes glioblastoma cell growth and invasion through activation of the mitogenic PI3K and ERK1/2 pathways which in turn upregulate COX-2 expression. Further studies involved the use of *si*RNA silencing COX-2 expression in ED273b glioblastoma cells is required for a better understanding of this novel mechanism.

Hypothesizing that the EP4 receptor is involved in modulation of glioblastoma cell proliferation and invasion provides the first evidence of a prominent role of the EP4 receptor in modulating of PGE_2 signaling. The activation of ERK1/2 and PI3K/Akt signaling pathways by PGE_2 leads to the induction of EGR-1 expression and TCF transcriptional activation. Our study is in

agreement with an early study showing that PGE_2-mediated EP4 receptor but not EP2 triggers activation of both PI3K and ERK1/2 (20). In addition, our study shows that signaling through the EP4 receptor enhances the increase of PGE_2 levels by increasing the expression of both COX-2 and mPGES-1 via PGE_2-mediated induction of TCF-4 and/or EGR-1 which is in agreement with other studies (21, 22). These findings may explain the increased levels of COX-2/PGE_2 and EP4 receptor we observed in high grade GBM. Future study is required to examine whether deletion or overexpression of the EP4 receptor alters PGE_2-induced glioblastoma tumor growth and development using EP4 knockout or transgenic mice.

Although the direct link between the EP4 receptor and the ERK1/2 and Akt signaling pathways was not established, our results demonstrate a critical role of EGFR signaling in GPCR-mediated stimulation of the ERK1/2 and PI3K/Akt cascades. We show that suppression of the EGFR tyrosine kinase activity by a specific inhibitor AG1478 abolishes activation of the ERK1/2 and Akt pathways as shown by others (23, 24). However, other studies have shown that GPCR mediated activation of ERK1/2 and PI3k/Akt cascades can occur without transactivating tyrosine receptor kinase (RTK) signaling (25) through a number of alterative mechanisms involving the stimulatory G_s subunit and cAMP (26), and phosphoinositide 3-kinase-γ (PI3Kγ) (27). Additional studies are required to analyse the role of EGFR signaling in GPCR-mediated activation of these signaling cascades. For example, future experiments should involve the use of cell

lines with siRNA silencing of G_s and/or PI3Kγ to show whether these molecules mediate activation of ERK1/2 and PI3K/Akt.

It has been noted that a downstream effector of PI3K/Akt signaling is the activation of the MAPKs superfamily members including ERK1/2, c-Jun NH_2-terminal kinase (JNK) and p38 kinases (28); however, the c-JNK and p38 pathways other than the ERK1/2 were not investigated in our study. Future studies will be initiated to examine the functional role of the JNK and p38 signaling pathways which may also play significant roles in GBM progression. Indeed, these pathways have been found to correlate with glioma invasion and progression (29, 30) and activation of the EP4 receptor has been shown to engage the p38 pathway in astrocytes (31).

The novel role of COX-2/PGE_2 signaling has recently been proposed as a cellular adaptation mechanism to the tumor hypoxic microenvironment in colorectal tumorigenesis (32). COX-2/PGE_2 and HIF-1α are the key components of inflammation and these factors act in concert to contribute to tumor cell proliferation, angiogenesis and invasion in the hypoxic tumor microenvironment (33). In Chapter 4, we further expand the knowledge of COX-2/PGE_2 signaling in glioma biology by determining that COX-2-derived PGE_2 plays a pivotal role within the hypoxic microenvironment in modulating HIF-1α transcriptional activity to enhance VEGF production and thus angiogenesis. Our hypoxia (1% O_2) results reveal that PGE_2 increases the HIF-1α transcriptional activity and protein expression via the EP4 receptor, thereby potentiating the VEGF secretion. However, two major concerns are noted in this study. First, we did not provide

evidence that *COX-2* is a hypoxia-responsive gene in glioblastoma tumor cells although it has been demonstrated in colorectal tumor cells by others (34). Future studies using a *COX-2* promoter in a luciferase reporter assay to measure the promoter activity in glioblastoma cells during hypoxia would help answer this question. Moreover, COX-2 upregulation by hypoxia has been shown to be mediated by NF-κB (35), peroxisome proliferator-activated receptors (PPARs) (36) and HIF-1α (37). Second, although our data support the conclusion that PGE_2 mediates the induction of HIF-1α transcription activity, protein expression and VEGF production under hypoxic conditions, future experiments using echinomycin (a small-molecule inhibitor of HIF-1 DNA binding activity) or *si*RNA silencing HIF-1α will determine whether PGE_2 has a direct effect on VEGF production under hypoxia conditions.

It is anticipated that developing a novel therapeutic strategy for EP4 receptor antagonism may be difficult due to the problem of EP-receptor function homogeneity. The significance of receptor homogeneity remains to be elucidated. It is likely that, for example, closely related receptor subtypes (EP2 and EP4) which are activated by PGE_2, are coupled to $G_\alpha S$ and signal through the same second messenger cAMP pathways (38). Similarly, binding studies show that selective EP4 antagonist ONO-AE3-208 retains notable affinity for EP3 receptor (39). In the context of tumor neovascularisation, the selective EP4 antagonist ONO-AE3-208 has been shown to reduce IL-1β-induced COX-2 mediated angiogenesis in the mouse cornea (40). Future studies will be undertaken to investigate the functional role of the EP4 receptor as well as the biological effects

of EP4 antagonists with respect to glioblastoma proliferation and invasion in the mouse xenograft model. This initial work, however, provides a foundation for future studies in this exciting field of PGE_2 research.

The discovery that cancer-initiating cells (CSCs) isolated from human glioblastoma and medulloblastomas (41) within a hypoxic vascular niche microenvironment (42) provides a window of opportunity for glioma treatment. We hypothesize that along with HIF-2α, PGE_2 may support and maintain CSCs and tumor endothelial cells within this vascular niche. In agreement with recent studies (43, 44), preliminary data in our laboratory have identified an important role for PGE_2-mediated stimulation of cellular dedifferentiation via Id (inhibitor of differentiation) protein regulated mechanisms. Further studies are required to clarify the PGE_2/Id protein relationship which may provide further insight into the maintenance of undifferentiated cells within these primitive tumors.

The significance of this thesis work is two-fold. First, in identifying the diarylprop-2-yn-1-one moiety compounds that induce cell cycle arrest and apoptosis via a non-COX/LOX target suggests that these 13-series compounds could be used as a lead compound for the development of a novel class of non-COX/LOX inhibitors for cancer prevention and treatment. Second, identification of PGE_2 signaling that requires the EP4 receptor in modulating glioblastoma cell growth and invasion provides an opportunity for the development of a novel targeted therapy by employing selective EP4 receptor antagonists which may ultimately lead to an alternate therapeutic strategy to current selective COX-2 inhibitors.

5.3. References

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